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Effects of Cardiotonic Steroids and Insulin on Sodium Pump Signaling

by

Shalini Gupta

Submitted to the Graduate Faculty as partial fulfillment of the requirements for the

Doctor of Philosophy Degree in

Biomedical Sciences

_________________________________________
Dr. Deepak K. Malhotra, Committee Chair

_________________________________________
Dr. Joseph I. Shapiro, Committee Member

_________________________________________
Dr. Sonia M. Najjar, Committee Member

_________________________________________
Dr. David R. Giovannucci, Committee Member

_________________________________________
Dr. Jiang Tian, Committee Member

_________________________________________
Dr. Patricia R. Komuniecki, Dean
College of Graduate Studies

The University of Toledo

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An Abstract of

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Cardiotonic steroids (CTS), including ouabain and marinobufagenin, have a natriuretic effect through their action on the Na\(^+\)/K\(^+\)–ATPase in the kidney. In addition to a role in inducing salt-sensitive hypertension, they are also implicated in insulin regulation. CarcinoEmbryonic Antigen Cell Adhesion Molecule (CEACAM) protein, a known mediator of insulin clearance via its interaction with the insulin receptor (IR), among its other functions, is also expressed in the kidney, and involved in the development of insulin resistance. We investigate a possible interaction between cardiotonic steroids and insulin in signaling through the sodium pump toward the development of insulin resistance and salt-sensitive hypertension. The sodium pump, insulin receptor, epidermal growth factor receptor (EGFR), and CEACAM1 are expressed on the basolateral aspect of polarize renal proximal tubule LLC-PK1 cells, where they can interact to facilitate intracellular signaling. Cardiotonic steroids and insulin, both of which can signal through the Na\(^+\)/K\(^+\)–ATPase, stimulated decreased plasmalemmal expression, with corresponding increase in early endosomal accumulation, of CEACAM1 protein. EGFR and IR showed similar changes with exogenous ouabain or
insulin stimulation, though these changes were not synergistic. Thus, in addition to its role in insulin clearance in the liver, CEACAM1 could also play a role in cardiotonic steroid-induced natriuresis and salt-sensitive hypertension in the kidney. This was supported by increased salt-sensitive hypertension in those mice lacking CEACAM2, a homologous and possibly functionally redundant protein present along with CEACAM1 in mice.

If as predicted, the incidence of diabetes does indeed increase in the future, associated diseases such as chronic renal failure may also have a greater impact. Taken together, these data provide molecular insight into a well-documented clinical association between chronic renal failure and insulin resistance. CEACAM, in addition to its extensively studied role in hepatic insulin clearance, is revealed as a novel player and potential therapeutic target in mediating the effects of increased cardiotonic steroids, as observed in patients with chronic renal failure, via the sodium pump.
There is an expression that, ‘the last miles are truly the hardest’. I can’t think of a more fitting way to sum up my thesis work. Those last miles were made bearable by my family—my parents, my two sisters, and my husband. I have seen them deal with the challenges in life with strength and grace and then turn around and support me when I needed it most. I am extremely thankful to have them in my life.

I would like to acknowledge and dedicate this thesis to my forever unconditional advocates: my parents.
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List of Abbreviations

α1 ................................Na+/K+–ATPase α1 Subunit
a.k.a. ..........................Also Known As
AngII ..........................Angiotensin II
ANOVA .....................Analysis Of Variance
ATP ..........................Adenosine Triphosphate

BSA ..........................Bovine Serum Albumin

C .................................Control
C2-7 ..........................Caveolin-1 Knock-Down LLC-PK1 Cells
Cav-1 ..........................Caveolin-1
CCT ...........................Cortical Collecting Tubule
CEACAM1 ..................CarcinoEmbryonic Antigen Cell Adhesion Molecule 1
CEACAM1-L ...............CarcinoEmbryonic Antigen Cell Adhesion Molecule 1 – Long
Isoform
CEACAM1-S ...............CarcinoEmbryonic Antigen Cell Adhesion Molecule 1 – Short
Isoform
CRF ..........................Chronic Renal Failure
cSrc ..........................total Src

DCT ..........................Distal Collecting Tubule
DLS ..........................Digitalis-Like Substance
DM ..........................Diabetes Mellitus
DMEM .....................Dulbecco’s Modified Eagle’s Medium

EDTA ......................Ethylenediaminetetraacetic Acid
EEA1 ..........................Early Endosomal Antigen 1
EGFR ..........................Epidermal Growth Factor Receptor
ENaC ..........................Epithelial Sodium Channel
ESRD .........................End-Stage Renal Disease

GAPDH ......................Glyceraldehyde 3-Phosphate Dehydrogenase

HTN ..........................Hypertension

I .................................Insulin
Ins.........................Insulin
IRβ .......................Insulin Receptor-β Subunit
IRS ........................Insulin Receptor Substrate

kDa.......................kilo Dalton

LLC-PK .....................Lily-Laboratory Culture-Porcine Kidney

M ..........................Marinobufagenin
MAPK ........................Mitogen-Activated Protein Kinase
MBG ........................Marinobufagenin
MDCK ........................Madin-Darby Canine Kidney
MR ..........................Mineralocorticoid Receptor
mRNA ........................messenger Ribonucleic Acid

NASH .......................Nonalcoholic Steatohepatitis
NHE ........................Na\(^+\)/H\(^+\) Exchanger

O ..........................Ouabain
Oub .........................Ouabain

P11 ..........................LLC-PK1 Cells With Vehicle Vector Transfection
PAGE .......................Polyacrylamide Gel Electrophoresis
PBS ........................Phosphate Buffered Saline
PCT ........................Proximal Collecting Tubule
PKC ........................Protein Kinase C
PMSF ........................Phenylmethylsulfonyl Fluoride
PNx ........................Partial Nephrectomy
pSrc ........................phosphorylated Src
PY-17 .......................Na\(^+\)/K\(^+\)–ATPase-α1 Knock-Down LLC-PK1 Cells

RAAS .......................Rennin-Angiotensin-Aldosterone System
ROD ........................Relative Optical Density

SBP ........................Systolic Blood Pressure
SDS ........................Sodium Dodecyl Sulfate
SEM ........................Standard Error of Mean
SIK1 ........................Salt-Inducible Kinase 1
siRNA ........................small interfering RNA
SS ...........................Sham Surgery

TAL ........................Thick Ascending Loop of Henle
TBS .........................Tris-Buffer Saline

WT ..........................Wild Type
List of Symbols

[ ] ......................... Concentration
i .......................... Intracellular

g.......................... Gravity
Chapter 1

Introduction

Chronic renal failure (CRF) affects approximately 19 million adult Americans, and its incidence is increasing rapidly (El-Atat et al., 2004b; Snyder and Pendergraph 2005). The annual mortality rate of end-stage renal disease (ESRD) is approximately 24 percent, and its incidence has doubled every decade since 1980. Moreover, the relative risk for mortality in end-stage renal disease subjects is consistent across different ages and genders (Tonelli et al., 2006). Diabetes Mellitus and hypertension are the two most prevalent syndromes implicated in chronic renal failure and its sequelae, accounting for approximately two-thirds of the cases of chronic renal failure and end-stage renal disease (Aneja et al., 2004; El-Atat et al., 2004a; Kobayashi et al., 2005; Snyder and Pendergraph 2005; Collins et al., 2010; Levey et al., 2010; Van Buren and Toto 2013). While a strong correlation between renal failure as well as cardiovascular and metabolic complications has been documented (Foley et al., 1998; Mandavi et al., 2013; Lastra et al., 2014), the molecular mechanisms underlying this correlation are still quite unclear. As patients with renal insufficiency have elevated levels of insulin (Liao et al., 2012) and cardiotonic steroids (CTS) (Gonick et al., 1998) which may contribute to these metabolic and
cardiovascular complications (Bagrov et al., 2005a, 2009b), we sought to investigate potential cross-talk between these two players and their roles in hypertension.

Cardiotonic steroids, i.e., digitalis-like substances (DLS), are steroid molecules which bind to the plasmalemmal sodium pump adenosine triphosphatase (Na\(^+\)/K\(^+\)–ATPase) (Bagrov et al., 2009b). CTS, which include ouabain and marinobufagenin (MBG), play important roles in cardiac and renal physiology (de Wardener, 1996; Friedman et al., 2002; Fedorova et al., 2010a). For instance, MBG induces natriuresis, and increases blood pressure in susceptible rat strains (Fedorova et al., 2002, 2005; Bagrov et al., 2009a). It is now well accepted that cardiotonic steroids induce signaling through the plasmalemmal Na\(^+\)/K\(^+\)–ATPase, particularly those residing in caveolae (Liu et al., 2003, 2005; Pierre and Xie 2006; Quintas et al., 2010).

Independent lines of evidence suggest that circulating cardiotonic steroids are important players in the pathophysiology underlying insulin resistance in many patients with chronic renal failure: Diabetes Mellitus is associated with an increase in cardiotonic steroid levels (in humans and in animal models), as well as with a dysfunctional Na\(^+\)/K\(^+\)–ATPase (Clerico and Giampietro, 1990; Chen et al., 1993; Straub et al., 1996). Of note, Type-I diabetic rats had higher levels of MBG as well as a greater degree of Na\(^+\)/K\(^+\)–ATPase dysfunction compared to Type-II diabetic rats (Bagrov et al., 2005a). Furthermore, oral glucose loading resulted in elevated levels of circulating CTS (Carroll et al., 2001). Based on the extensive literature concerning cardiotonic steroids, as well as data from our laboratory, we propose that circulating cardiotonic steroids and insulin share a common signaling cascade. This signaling may contribute in part to the
pathophysiology of hypertension and insulin resistance seen clinically in chronic renal failure patients (El-Atat et al., 2004a).

In order to investigate this central hypothesis, we utilized an *in vitro* system to study the effects of ouabain and insulin on key molecules important in insulin signaling. The altered endocytosis of such molecules in response to ouabain or insulin stimulation can lead to altered insulin clearance, and thus contribute to the development of insulin resistance. We also investigated the development of salt-sensitive hypertension in a genetically modified mouse model characterized by an altered insulin response and energy balance.
Chapter 2

Literature Review

Hypertension, or high blood pressure, is present in 50% of patients with Diabetes Mellitus (DM), and contributes significantly to cardiovascular disease and other morbidities and mortalities in these individuals. According to a 2011 report, 25.8 million people, 8.3% of the United States population, have DM (Center for Disease Control and Prevention, 2011). Worldwide there are 240 million people with DM, with a projected 439 million by the year 2030 (Shaw et al., 2010). As mentioned earlier, hypertension is a frequent co-morbidity in patients with diabetes, especially with type II DM or type I diabetes associated with renal disease (Szuszkiewicz-Garcia and Davidson 2014).

Hypertension is a chronic medical condition in which the systemic arterial blood pressure is elevated, usually defined to be systolic (SBP) and diastolic pressures greater than 140 mmHg and 90 mmHg, respectively. It is classified as either Primary (a.k.a. Essential) or Secondary (a.k.a. Inessential) hypertension. Ninety to ninety-five percent of cases cannot be attributed to a medical condition and are termed Primary hypertension. The remaining 5-10% of cases is caused by an identifiable medical condition often affecting the kidney, heart or endocrine system, with the most common cause of Secondary hypertension being renal parenchymal disease (Yerram et al., 2012). A recent
meta-analysis of patients with DM and pre-diabetes suggested that a SBP of 130 to 135 mmHg may be optimal and markedly decrease risks of stroke and other morbidities in these individuals (Bangalore et al., 2011; Garcia-Touza and Sowers 2012).

Currently one third of the US population is hypertensive, representing a three- to six-fold increase since the early 1900s (Johnson et al., 2005). The National Health and Nutrition Examination Survey (NHANES) conducted from 2005 through 2008 estimated that hypertension affects up to 65 million adults in the United States (Egan et al., 2010). The evidence is overwhelming that current intake of salt contributes in a major fashion to the current ‘epidemic’ of hypertension and justifies public health efforts to reduce salt intakes, particularly in commercial food items (Al-Awqati 2006): 85% of salt-intake is already contained in commercial food items, leaving only 15% under the direct control of the patient (Sanchez-Castillo et al., 1987; Brown et al., 2009). These are some of the factors that play an important role in the development of hypertension associated with insulin resistance, and explored in this thesis.

2.1 An Association Between Hypertension and Chronic Renal Failure

Chronic hypertension leads to end-organ damage, including in the kidneys (Van Buren and Toto 2013). Speculation about this connection is long-standing, dating back at least to Richard Bright’s suggestion in 1827 that chronic kidney disease may be due to hypertension (Bright 1827). However, only in the last several decades has altered kidney function, particularly due to high salt diet, been identified as one cause of hypertension.
(He and MacGregor 2009; Lubanski and McCullough 2009). Studies by Allen and Dahl helped formulate the theory that hypertension might relate to a defect in sodium excretion (Allen 1920, Dahl 1960; Dahl 2005). Arthur Guyton was the first to provide a quantitative basis for the relationship between blood pressure and natriuresis, i.e., a pressure-natriuresis (Guyton et al., 1972). According to this curve, an increased salt load shifts the relationship curve to the right, thereby requiring higher blood pressure to enable the kidneys to excrete the excess sodium loads in an attempt to return to the baseline steady-state pressure (Guyton 1961; Guyton and Coleman 1999). Given the difficulty in conducting relevant long-term observational studies in humans according to the INTERSaLT studies (Elliott et al., 1989, 1996; Titze and Ritz 2009), numerous in vivo models including salt-sensitive animals have been extensively utilized (Elliott et al., 2007).

2.2 Salt-Handling in Hypertension

Modern day humans evolved consuming approximately 1 g salt/day (Eaton and Konner 1985). In many Western acculturated societies, life-long consumption of salt range from approximately 4 to 10 g/day which far exceeds need, and has been linked with the incidence of hypertension across populations (Intersalt Cooperative Research Group 1988). The molecular mechanism by which salt raises blood pressure is unknown. Since the initial work by Guyton and Dahl, the kidney’s crucial role in regulating blood pressure via sodium excretion has been further elucidated (Cowley and Roman 1996;
Hall et al., 1990; Wang et al., 2000; Rodriguez-Iturbe et al., 2007; Franco et al., 2008; Udani et al., 2011). Briefly, the renal glomerular apparatus filters over 170 liters of plasma (containing 23 moles of salt). As such, the kidneys must reabsorb 99.5% of the filtered salt to maintain salt homeostasis on a typical 100 mEq sodium diet, which it accomplishes by an integrated system of ion channels, exchangers, and transporters along the nephron, the functional unit of the kidney. Typically, 60% of the filtered sodium is reabsorbed almost immediately by the proximal collecting tubule (PCT) of the nephron largely by apical Na$^+$/H$^+$ Exchanger (NHE) and basolateral Na$^+$/K$^+$/ATPase. 30% of sodium is reabsorbed in the thick ascending limb of Henle (TAL) by the Na$^+$/K$^+$/2Cl$^-$ co-transporter. Seven percent is reclaimed by Na$^+$/Cl$^-$ cotransport in the distal convoluted tubule (DCT). Finally, the last 2% is reabsorbed via the epithelial Na$^+$ channels (ENaC) in the cortical collecting tubule (CCT).

Though the CCT reabsorbs only a small fraction of sodium, it is considered the principal site involved in the regulation of net salt balance, and itself is highly regulated by the renin-angiotensin-aldosterone system (RAAS) (Johnson et al., 2008). Decreased delivery of sodium to the thick ascending limb of Henle leads to increased secretion of the aspartyl protease rennin, which results in increased formation of the short peptide hormone angiotensin II (AngII). AngII binds to its specific G protein-coupled receptor in the zona glomerulosa of adrenal cortex, inducing secretion of aldosterone, the principal mineralocorticoid steroid (MR) hormone. Aldosterone binding to MR, a nuclear hormone receptor in principal cells of the distal nephron, and initiates a sequence of events leading to increased ENaC activity and salt reabsorption. The pathophysiologic link between salt and blood pressure is predictable from the relationship between salt and vascular volume.
homeostasis. Increased salt load necessitates increased water reabsorption in order to maintain physiologic plasma sodium concentration at or near 140 mM. The resulting increased intravascular volume augments venous blood return to the heart, thereby raising cardiac output and leading directly to elevated blood pressure. Conversely, impaired reabsorption of salt reduces blood volume and blood pressure (Lifton et al., 2001; McDonough et al., 2003; Johnson et al., 2008).

The CNS, the peripheral vasculature and the renal medulla link salt sensing with the salt-sensitive control of BP and kidney function. Short-term changes in dietary salt and CNS infusions stimulate salt-sensing pathways within the brain that initially mediate large increases in sympathetic nervous system activity that constrict the peripheral vasculature and raise BP. The more clinically relevant phenomenon of long-term salt sensitivity involves central sodium sensors that also stimulate a pathway that has a slow pressor action. The slow pathway involves the activation of central aldosterone, mineralocorticoid receptors, ENaCs, endogenous ouabain and angiotensin II-type 1 receptors and, in the periphery, raises circulating CTS. Chronic elevation of circulating endogenous ouabain (and possibly other CTS) amplifies existing sympathetic tone, augments the contractile function of the peripheral vasculature, suppresses nitric oxide-related signaling in the renal medulla and resets kidney function to help maintain the elevated blood pressure.
2.2.1 Salt-Handling in Hypertension—Role of the Proximal Collecting Tubule

Continued work in animals has established the proximal tubule as an important determinant of the alteration in the pressure-natriuresis relationship in “sodium-sensitive” hypertension (Rapp 1982; Strazzullo et al., 2003; Burnier et al., 2006). Salt sensitivity of blood pressure is defined as the interindividual difference in the blood pressure response to changes in dietary sodium chloride intake; it implies an alteration in the slope of the pressure-natriuresis relationship, and was demonstrated in numerous studies involving humans (Barba et al., 1996; Chioléro et al., 2000). These studies also provide support for the proximal renal sodium handling to be an important determinant of the alterations in the pressure-natriuresis relationship that occurs in patients with salt-sensitive hypertension, independent of changes in renal hemodynamics (Jaitovich and Bertorello 2010a).

Furthermore, increased blood pressure has been shown to attenuate expression of apical NHE3, an isoform distinctly expressed in renal PCT, as well as inducing rapid, reversible inhibition of renal cortex basolateral Na\(^+\)/K\(^+\)—ATPase activity (Zhang et al., 1998). Removal of NHE3 from the apical brush border and inhibition of basolateral Na\(^+\)/K\(^+\)—ATPase would diminish PCT sodium reabsorption that drives increased salt delivery to the macula densa to affect autoregulation during hypertension (Magyar et al., 2000; Burnier et al., 2006; Wang et al., 2009).
2.3 Cardiotonic Steroids: A Brief Overview

In 1785, William Withering first described the properties and medical use of digitalis (Withering 1785). It was not until 1799 when the importance of the direct action of digitalis on the heart was considered (Ferriar 1799). Over the years, not only has the therapeutic potential of digitalis in the heart been well elucidated, additional roles for it in the kidney, and placenta been disclosed.

According to the most widely accepted mechanism of action, digitalis binds and inhibits the Na$^+$/K$^+$–ATPase, thus indirectly raising the intracellular Ca$^{2+}$ concentration ([Ca$^{2+}]_i$). Therapeutic concentrations of digitalis compounds produce a moderate enzyme inhibition (about 30%). The remaining non-inhibited enzymes work faster to restore the ionic balance. However, a lag due to the inhibited enzymes causes a temporary increase of [Na$^+]_i$, and therefore [Ca$^{2+}]_i$ through accelerated Na$^+$–Ca$^{2+}$ exchanger activity, and ultimately increases myocardial contractility (Blaustein 1974). However, when the concentration of digitalis compounds reaches toxic levels, sodium pump inhibition is too high (>60%). The concentrations of Na$^+$ and K$^+$ decrease to the extent that the restoring of normal levels during diastole is not possible before the next depolarization. Then, a sustained increase of [Na$^+]_i$ and, thus of [Ca$^{2+}]_i$ gives rise to toxic effects (i.e., arrhythmia) of these compounds (Merelo et al., 2000).
2.3.1 Digitalis-Like Substances in Chronic Renal Failure

Digitalis-like substances such as marinobufagenin and ouabain (Figure 2-1) are endogenous cardiotonic steroids found to be elevated in circulation of patients with chronic renal failure (Gonick et al., 1998) and may contribute to the hypertension and insulin resistance often observed in these patients (Hamlyn et al., 1998; Manunta et al., 2009; Kolmakova et al., 2011). Elevated MBG levels have also been linked to Diabetes Mellitus (Bagrov et al., 2005b).

![Diagram of Ouabain and Marinobufagenin](image)

**Figure 2-1.** Cardiotonic Steroids. A) Ouabain, B) Marinobufagenin.

Cardiotonic steroids are synthesized from cholesterol in the adrenal cortex and hypothalamus (Dmitrieva et al., 2000; Komiyama et al., 2001; el-Mastri et al., 2002; Schoner 2002; Murrell et al., 2005). Their production and secretion is regulated by multiple pathological and physiological stimuli, including adrenocorticotropin, angiotensin II, and epinephrine (Laredo et al., 1997; Fedorova et al., 1998). Ouabain and marinobufagenin, a natriuretic and vasoconstrictor, stimulate signal transduction by selectively binding the $\text{Na}^+/\text{K}^+$–ATPase receptor (Lopatin et al., 1999; Aydemir-Koksoy
et al., 2001; Tian et al., 2006). In particular, these endogenous cardiotonic steroids mediate an increase in blood pressure following the administration of a sodium load. In addition to Angiotensin II, aldosterone, the atrial peptides, and nitric oxide, circulating endogenous ouabain has been linked with variations of electrolyte balance, renal function and hypertension (Manunta et al., 2009; Blaustein et al., 2012; Hamlyn and Blaustein 2013). Consistent with this proposal, people with hypertension have increased—albeit relatively small—levels of sodium in plasma (de Wardener et al., 2004). The proximate cause of increased levels of cardiotonic steroids in hypertensive patients is an increased level of sodium in the CSF (Schoner and Scheiner-Bobis 2007), with the latter mirroring the sodium level in the plasma (Lichtstein and Rosen 2001). Chronic elevation of brain sodium promotes sustained hypertension mediated by central endogenous ouabain and the Na$^+$/K$^+$ pump $\alpha_2$ catalytic subunit (Lorenz et al., 2008; Van Huysse et al., 2011). The intermediary pressor mechanism in the brain involves aldosterone biosynthesis, activation of mineralocorticoid receptors and increased epithelial sodium channel activity. In the periphery, elevated plasma CTS raise contractility and blood pressure by augmentation of sympathetic nerve responses, increasing arterial Ca$^{2+}$ signaling and blunting nitric oxide production in the renal medulla and collecting ducts (Dostanic et al., 2005). Among the virtues of this model is its ability to explicate the increased urinary levels of MBG subsequent to salt-loading in the Dahl rat, a salt-sensitive hypertensive animal model (Fedorova et al., 2001). Of note, the Dahl rat has a mutation in the alpha-1 subunits of its Na$^+$/K$^+$–ATPase (Fedorova et al., 2000). During renal insufficiency, sodium pump-mediated actions of MBG could regulate downstream signals including the internalization of epidermal growth factor receptor (EGFR) and possibly insulin receptor.
Endogenous ouabain in humans is modulated by dietary salt and chronic volume status. Endogenous ouabain is linked significantly with vascular function in hypertension and likely impacts the pathogenesis of heart and renal failure (Haddy 2006; Manunta et al., 2010). Moreover, the molecular mechanism of endogenous ouabain-linked hypertension involves the sodium pump/sodium-calcium exchanger (Blaustein et al., 2009; Blaustein and Hamlyn 2010).

Interestingly, recent reports have demonstrated higher MBG levels in women with pre-eclampsia (PE), another disease associated with hypertension and gestational diabetes (Averina et al., 2006; Lamarca et al., 2006; Fedorova et al., 2010b). Based on these and other clinical observations, DLS could be potential contributors of hypertension and insulin resistance in these diseases. MBG but not ouabain shows four-fold increase in pre-eclamptic placentae (Uddin et al., 2012). Digibind, a therapeutic digoxin antibody which binds CTS, lowers blood pressure and reverses Na\(^+/\)K\(^+\)–ATPase inhibition in patients with pre-eclampsia (Goodlin 1988; Adair et al., 2009).

2.4 Relationship between Blood Pressure, and Salt Intake—The J-Curve

A significant impact of salt on blood pressure in many but not all individuals has lead to the recommendation of a population-wide reduction in sodium intake. Even in salt-sensitive individuals, the depressor effects of a low-sodium diet are offset to a variable degree by reflex increases in many variables including plasma rennin and aldosterone levels, sympathetic nerve activity, plasma insulin and glucose, and serum
lipids (Hamlyn and Blausteine 2013). In individuals with average sodium intake of less than 4 grams/day, there is generally an inverse association of intake and adverse outcome (Stolarz-Skrypek et al., 2011). In contrast, an average sodium intake of above 5 grams/day correlates with a more direct effect on blood pressure. This overall relationship between salt intake with cardiovascular outcomes is ‘J-shaped’, and is also observed in other circumstances. For instance, a J curve relates maternal salt intake during pregnancy with low nephron number in offspring (Koleganova et al., 2011). In Diabetes Mellitus type II; moderate lowering of blood pressure can be beneficial, whereas a more aggressive correction of blood sugar to target values near normal increases mortality (Gerstein et al., 2011).

2.5 \(\text{Na}^+/\text{K}^+\text{–ATPase} \text{ – Structure and Function}\)

The \(\text{Na}^+/\text{K}^+\text{–ATPase}\) is a member of the P-type ATPase superfamily (Lingrel et al., 1994b; Lingrel and Kuntzweiler 1994; Lingrel et al., 1998). The pump is a transmembrane protein composed of two noncovalently linked \(\alpha\) and \(\beta\) subunits (Lingrel et al., 1994a; Sweadner, 1989) (Figure 2-2). The \(\alpha\) subunit (approximately 112 kDa and 1012 amino acids) consists of ten transmembrane domains, with both the C- and N-terminal ends into the cytoplasmic side (Jorgensen and Andersen 1998). The \(\beta\) subunit (approximately 55 kDa and 300 amino acids) has one transmembrane segment, a short N-terminal end towards the cytoplasmic side and a large extracellular segment, bearing
three disulfide bridges (Kirley 1989). The sodium pump α and β subunits are the “catalytic subunit” and “regulatory domain,” respectively, with the latter being the limiting one for the formation of the functional enzyme. The α subunit contains the ATP, digitalis, and other ligand binding sites, and is essential for the assembly of the functional Na⁺/K⁺–ATPase enzyme (Skou 1982). Four isoforms of the α subunit and three isoforms of the β subunit have been identified and functionally characterized, and are expressed in a tissue-specific manner (Blanco and Mercer 1998; Besirli et al., 1997; Lingrel et al., 1994a, b; Shull et al., 1986; Sweadner 1989). The α₁ subunit is found in all cells including hepatocytes and renal cells, unlike the α₂, α₃ isoforms (expressed in skeletal muscle, neural tissue, and cardiac myocytes), and α₄ isoform (expressed only in the testes) (Blanco and Mercer 1998; Blanco et al., 2000; Lingrel et al., 1988, 1990). Among the β isoenzymes, β1 is present in all tissues, being the main isoform found in mammal kidney. The β2 and β3 isoforms have been isolated in the brain (Shyjan et al., 1990; Lingrel 1992; Yu et al., 1997). Any of the α subunits can be assembled with any of the β subunits, thus giving rise to different enzyme complexes (Crambert et al., 2000).
The Na\(^+\)/K\(^+\)–ATPase has been extensively studied as an ion pump since its discovery in 1957 (Skou 1957). Research since then has established that this enzyme uses ATP as an energy source to transport 3 Na\(^+\) out in exchange for 2 K\(^+\) in across the plasma membrane to maintain cell’s electrical polarity (Lingrel et al., 1994a; Lingrel and Kuntzweiler 1994; Sweadner, 1989). In addition to binding ATP, cardiac Na\(^+\)/K\(^+\)–ATPase is the functional receptor for the inotropic effects of digitalis (Schwartz et al., 1988). Inhibition of the enzyme by digitalis in the heart raises [Ca\(^{2+}\)\(_i\)] and myocardial contractility, and thus facilitates the clinical use of digitalis drugs in the therapy of congestive heart failure. In particular, the \(\alpha\) subunit has an extracellular binding site for digitalis and other cardiotonic steroids including ouabain and marinobufagenin (Askari 2000; Xie and Askari 2002). The different \(\alpha\) isoforms have varying affinities for ouabain and MBG.

In addition to its classical role, the sodium pump has been more recently proposed to function as a receptor or plasma membrane enzyme for targeted intracellular signaling (Aizman et al., 2001; Schoner and Scheiner-Bobis 2005; Tian et al., 2006; Xie 2001; Xie and Askari 2002). Cumulative studies from our and other laboratories show that binding of cardiotonic steroids to Na\(^+\)/K\(^+\) pump affects not only ion flux, but also protein trafficking, gene expression, cell attachment and proliferation, formation of tight junctions, and modification of immune response (Aydemir-Koksoy et al., 2001; Aydemir-Koksoy and Allen 2001; Belusa et al., 2002; Rajasekaran et al., 2001; Vega et al., 2002; Baudouin-Legros et al., 2003; Huang et al., 1997 a, b; Liu et al., 2000, 2002; Mohammadi et al., 2001). Cardiotonic steroids can selectively bind the Na\(^+\)/K\(^+\) pump–\(\alpha1\) subunit to inhibit pump activity (Fedorova et al., 2001). Conversely, CTS-induced signal
transduction via the Na\textsuperscript{+}/K\textsuperscript{+}–ATPase molecule leads to tyrosine kinase Src activation, which in turn phosphorylates and activates multiple signaling cascades including Ras/Raf and PKC pathways (Haas et al., 2000, 2002) (Figure 2-3). These elevated cardiotonic steroids signaling through the Na\textsuperscript{+}/K\textsuperscript{+}–ATPase can contribute to manifestation of the above mentioned pathologic disease symptoms.

![Figure 2-3. Potential role of cardiotonic steroids and insulin in a proposed schematic of cross-talk between Na\textsuperscript{+}/K\textsuperscript{+}–ATPase and insulin receptor.](image)

### 2.6 Insulin Action

Glucose homeostasis is maintained by insulin acting on its receptor in target tissues including the liver, kidney, muscle, and adipose tissue. Secondary to increased blood glucose level, insulin is secreted from pancreatic beta cells and signals through the insulin receptor (IR) to reduce glucose synthesis and stimulate glucose uptake and storage (as glycogen in liver and muscle, and as triglyceride in adipose tissue), thereby returning
blood glucose to normal levels in the bloodstream (Figure 2-4) (Youngren 2007). Briefly, the insulin receptor is made up of alpha and beta subunits that phosphorylate upon binding of insulin (Lee and Pilch 1994). The binding of insulin to its receptor, a tetrameric transmembrane protein with an intracellular tyrosine kinase domain, triggers receptor activation and autophosphorylation of the IR β subunits, and initiates a downstream signaling cascade (Figure 2-5) (Choice et al., 1998). The resultant ligand-induced signal transduction is mediated by the activation of endogenous signaling molecules (including IRS-1, -2, Shc, and CEACAM1), and ultimately facilitates glucose transport into the cell (White and Kahn 1994). Additionally, the insulin–receptor complex is internalized into geometrically latticed clathrin-coated pits, which pinch off from the

![Figure 2-4. Schematic outlining the fate of insulin and insulin receptor.](image)
surface membrane to form clathrin-coated vesicles (Hari and Roth 1987; Carpentier et al., 1993). Within the vesicle, insulin is degraded in a low pH environment, and the receptor is recycled to the plasma membrane (Figure 2-4) (Backer et al., 1990).

Figure 2-5. Schematic of insulin receptor and CEACAM1 interaction and endocytosis.

In addition to these clathrin-coated pits, caveolae have more recently been implicated in regulation of insulin receptor trafficking (Cohen et al., 2003). Caveolae are 60 to 90 nm invaginations in the plasma membrane (Rothberg et al., 1992), and lined by caveolin (previously known as VIP21, vesicle integral protein of 21 kDa) (Kurzchalia et al., 1994). Interestingly, caveolae are almost exclusively found on the basolateral side of polarized epithelial cells, with minimal presence on apical side (Breton et al., 1998; Vogel et al., 1998; Brown 2000). Reduction in an important caveolar protein, namely caveolin-1 (cav-1), blunted insulin receptor internalization. Moreover, the caveolin-1
scaffolding domain shields IR from proteosomal degradation (Cohen et al., 2003). Caveolin-1, most abundantly expressed in adipocytes, is also clearly present at lower levels on cell surface of hepatocytes and may play a major role in insulin signaling in target tissues (Pol et al., 1999; Calvo et al., 2001; Babis et al., 2004). Loss of cav-1 is not sufficient to produce fulminant diabetes, though its absence can produce insulin-resistance. Caveolin-1 null mice have markedly reduced IR levels in adipocytes, with blunted insulin-inducible activation of downstream targets such as PKB/Akt and GSK-3β (Cohen et al., 2003). Interestingly, a subset of patients with severe insulin resistance was found to have mutations in caveolin-binding motif of insulin receptor (Cohen et al., 2003). Overall, impaired receptor recycling can lead to hyperinsulinemia and the development of insulin resistance.

While the classic model of insulin action focused on the liver and skeletal muscle, insulin receptor is also present in the kidney—now also considered a target organ for insulin action (Skott et al., 1989; Hale and Coward 2013). In the kidney, insulin enhances sodium re-absorption in the diluting segment of the distal nephron, in part through increased expression of sodium transporters like the ENaC, with consequent decrease in sodium excretion (Song et al., 2006). Hyperinsulinemia-mediated sodium retention could potentially contribute to the genesis of hypertension via increased activation of NHE activity in the proximal tubule as well as through the effects of ENaC more distally. However, Tiwari and others also showed increased systolic blood pressure with increased sodium retention with kidney-specific IR knockout (Tiwari et al., 2008). Though paradoxical, these findings could suggest that decreased number or efficiency of
signaling of existing IR in the kidney contribute similarly to the elevated systolic blood pressure and increased sodium re-absorption.

2.6.1 CEACAM1 – Structure and Function

CarcinoEmbryonic Antigen Cell Adhesion Molecule 1 (CEACAM1; formerly known as biliary glycoproteins BGP, C-Cam, CD66a, or MHVR), a tumor-suppressor, is a plasma membrane glycoprotein (Zebhauser et al., 2005; Nouvion and Beauchemin 2009). The ~120kDa glycosylated protein is encoded by a single gene (Ceacam1 encoding 9 exons; Figure 2.6B) in the rat (519 amino acids) and human genomes (located on Chromosome 19; Figure 2.6A). CEACAM1 is a transmembrane protein with an extracellular region containing from one to four immunoglobulin loops, and an intracellular region containing serine and tyrosine phosphorylation sites (Najjar et al., 1993). It is highly expressed in kidney and liver as two splice-variants that differ by a 61 amino acid sequence in the C-terminal cytoplasmic domain (Figure 2.6C; Prall et al., 1996; Cheung et al., 1993). While the truncated form lacks all phosphorylation sites (CEACAM1-4S), the full-length form (CEACAM1-4L) contains serine (Ser\textsuperscript{503}) and two tyrosine (Tyr\textsuperscript{488}, Tyr\textsuperscript{513}) phosphorylation sites (Najjar et al., 1993; Najjar 2002). CEACAM1 was originally identified as a substrate of the insulin receptor (Rees-Jones and Taylor, 1985), and later also of EGFR (Abou-Rjaily et al., 2004). In hepatocytes, CEACAM1 is a substrate of the IR tyrosine kinase; it is phosphorylated by cAMP-dependent serine kinase at Ser\textsuperscript{503} in the absence of insulin (Najjar 1998). Ser\textsuperscript{503}
phosphorylation is required for insulin-dependent phosphorylation of the Tyr\(^{488}\) residue by the insulin receptor tyrosine kinase. Though it is not phosphorylated by IR tyrosine kinase, intact Tyr\(^{513}\) is required for phosphorylated CEACAM1 to activate serine kinase in response to insulin (Najjar et al., 1995; Poy et al., 2002b).

Figure 2-6. Localization (A) and schematic representation of CEACAM1 mRNA (B), and protein (C).

While the function of CEACAM1 remains elusive, four proposed roles include (Kuespert et al., 2006): 1) cell-cell adhesion (Öbrink 1997), 2) tumor suppression (Lu et al., 1998), 3) down-regulation of the mitogenic effects of insulin (Formisano et al., 1995; Soni et al., 2000), and 4) up-regulation of receptor-mediated insulin endocytosis and degradation (Li Calzi et al., 1997; Najjar et al., 1998) to mediate hepatic insulin clearance (Choice et al., 1998). Phosphorylation of CEACAM1 is followed by two- to three-fold increase in IR internalization into the cell (Choice et al., 1998).
With respect to insulin action and clearance, Shc facilitates interaction of phosphorylated CEACAM1 with insulin receptor to induce receptor-ligand complex endocytosis via clathrin-coated pits (Rapoport et al., 1997; Najjar et al., 2002; Poy et al., 2002a). Inhibiting CEACAM1 expression in H4IIE hepatoma cells was associated with a decrease in IR complex internalization (Formisano et al., 1995). Consequently, reduced surface IR expression due to impaired receptor recycling or degradation can impair insulin clearance in the liver (Najjar et al., 1998). Inactivation of CEACAM1 in mice prevents the IR-CEACAM1-Shc complex to internalize and results in impaired insulin clearance, and subsequently hyperinsulinemia and insulin resistance (Poy et al., 2002b; Park et al., 2006). A similar phenotype is seen in rats with decreased level of CEACAM1 (Wisloff et al., 2005). Thus, CEACAM1 is a crucial regulator of insulin signaling and clearance in insulin-target tissues. More recently, CEACAM1 has been implicated in impaired bone remodeling due to decreased osteoclastogenesis and resulting increased bone mass (Huang et al., 2010). CEACAM1-deficient mice with insulin resistance developed key hepatic changes consistent with nonalcoholic steatohepatitis (NASH) in humans in response to a high fat diet (Cong et al., 2008; Lee et al., 2008; Ghosh et al., 2010; Najjar and Russo 2014).

CEACAM1 is present in the kidney, including in polarized renal epithelial cells of MDCK cells (Sundberg and Öbrink 2002). Similarly, altered CEACAM1 activity in patients with renal insufficiency could contribute markedly to the impaired insulin and blood pressure regulation in these individuals.
2.6.2 CEACAM2 – Structure and Function

While CEACAM1 has been extensively studied, not very much is known about CEACAM2. First identified on chromosome 7 of BALB/c and C57BL/6 mice, Ceacam2 (formerly named Bgp2) is nearly 25 kilobases long in comparison to the 17-kilobase Ceacam1 gene (Nedellec et al., 1994; Han et al., 2001). There is only one Ceacam1 gene expressed in human and one in rat, while two homologous genes (Ceacam1 and Ceacam2) have been identified in the mouse (Figure 2-7) (Han et al., 2001).

![Figure 2-7. Comparison of Ceacam1 and Ceacam2 genes.](image)

Ceacam1 and Ceacam2 also differ greatly in their tissue distribution in mice. Ceacam1 mRNA is highly abundant, including in the liver, small intestine, prostate, and spleen. Conversely, Ceacam2 mRNA has a tissue-specific distribution limited to the mouse kidney, testis, uterus, crypt epithelia of intestinal tissue, and to a lesser extent, spleen (Robitaille et al., 1999; Han et al., 2001; Zebhauser et al., 2005). More recently, Najjar reported Ceacam2 mRNA in the mouse hypothalamus, where it is involved in the regulation of energy balance (Heinrich et al., 2010; Patel et al., 2012). While the gene sequences between Ceacam1 and Ceacam2 are similar, their two corresponding products...
may or may not exhibit functional redundancy depending on the target tissue. For instance, CEACAM2 does not act as a cell adhesion molecule (Robitaille et al., 1999). Functional overlap in tissues expressing both CEACAM1 and CEACAM2 includes the liver, where both are involved in regulating insulin sensitivity (Poy et al., 2000b; Heinrich et al., 2010). Ceacam1 and its homologue Ceacam2 are both expressed in the kidney. Similar to the spontaneous hypertension observed with reduced Ceacam1 expression, mice lacking Ceacam2 may also exhibit renal impairment.

2.7 Do Cardiotonic Steroids and Insulin Signaling Cross Paths?

That ouabain and insulin signal through the $\text{Na}^+/\text{K}^+-\text{ATPase}$ and IR, respectively, is well established. This proposal explores whether they may share a common signaling mechanism whose effects may help dissect the metabolic complications observed in chronic renal failure. In addition to the suggestive evidence discussed earlier—the correlation of increased CTS levels and a dysfunctional $\text{Na}^+/\text{K}^+-\text{ATPase}$ with Diabetes Mellitus, increased levels of CTS subsequent to oral glucose loading, as well as the increased levels of MBG in Type I vs. Type II diabetic rats—oral glucose loading in the presence of anti-MBG antibodies has been shown in rat models leads to higher levels of insulin and glucose (Bagrov et al., 2007).

Evidence is emerging to support the hypothesis that ouabain and insulin may indeed have common effectors. Ouabain induced glycogen synthesis in skeletal muscle (Kotova et al., 2006), while insulin stimulated sodium pump activity (Al-Khalili et al.,
Féraillé and colleagues reported insulin-induced phosphorylation of the Tyr^10 residue on the alpha-1 subunit to stimulate Na\(^+\)/K\(^+\)–ATPase activity in renal proximal tubule cells (Féraillé et al., 1999). One proposed mechanism of action is outlined in Figure 4. As illustrated, cardiotonic steroids can signal through the sodium pump to activate the tyrosine kinase Src, which, in turn, can transactivate epidermal growth factor receptor to activate the ras/MAPK pathway. ERK1/2 mediates ouabain- and insulin-stimulated phosphorylation of Na\(^+\)/K\(^+\)–ATPase-\(\alpha_1\) subunit in human skeletal muscle cells (Al-Khalili et al., 2004; Kotava et al., 2006). This response was abolished in response to inhibition of MEK1/2, an upstream kinase of ERK1/2. EGFR can interact with the sodium pump and insulin receptor via Src and Shc, respectively (Abou-Rjaily et al., 2004). CEACAM1 is also a substrate for EGFR, and mediates EGF-dependent cell proliferation by its ability to bind and sequester Shc (Abou-Rjaily et al., 2004). Moreover, while insulin action through clathrin-coated pits is well documented, ouabain-mediated signaling via sodium pump in clathrin-coated pits (Liu et al., 2004) and insulin action via caveolar pits has also been recorded (Cohen et al., 2003). EGF-induced phosphorylation of caveolin-1 at the Tyr14 residue stimulates caveolae formation of epithelial cells (Orlichenko et al., 2006). In light of these, we suggest that cardiotonic steroids and insulin share a common pathway to mediate signaling.
Chapter 3

Effect of Marinobufagenin on CEACAM1 Expression in the Rat Kidney

by

Shalini Gupta¹ and Joseph I Shapiro¹,²

From

The Departments of Medicine¹ and of Physiology and Pharmacology². University of Toledo College of Medicine, Toledo, Ohio, 43614

Address correspondence to:

Joseph I. Shapiro, MD, Mail Stop #1186 Health Science Campus, University of Toledo College of Medicine, 3000 Arlington Avenue, Toledo, Ohio 43614-2598.

Phone: 419-383-6030;

FAX: (419)383-6244;

E-mail: Joseph.Shapiro@utoledo.edu

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3.1 Abstract

Clinically, patients with chronic renal failure exhibit an increased incidence of cardiovascular and metabolic complications including hypertension and insulin resistance. Insulin and cardiotonic steroids, including ouabain and marinobufagenin, are both elevated in chronic renal failure, and classically act through the insulin receptor and caveolar Na⁺/K⁺–ATPase, respectively. CarcinoEmbryonic Antigen Cell Adhesion Molecule (CEACAM) 1, a tumor-suppressor protein, has been well established as a key mediator in regulation of IR recycling and development of insulin resistance. We sought to investigate a role for CEACAM1 in marinobufagenin-mediated signaling through the sodium pump in an experimental rat model of chronic renal failure and modified cell lines with attenuated expressions of Na⁺/K⁺–ATPase-α1 subunit, and caveolin-1. We report attenuated CEACAM1 protein, but not mRNA, expression in liver of partially nephrectomized rats with elevated plasma marinobufagenin, along with decreased cell-surface expressions of CEACAM1, and insulin receptor β in liver H4IIE cells treated with exogenous MBG. These data suggest the effects of CEACAM1, at least in part, to be modulated by post-translational modification. CEACAM1, also expressed in renal LLC-PK1 cells, co-localizes with the α1-subunit of Na⁺/K⁺–ATPase, and is endocytosed along with the sodium pump in response to ouabain. Immunofluorescence studies showed this response to be attenuated in LLC-PK1 cells with knock-down expression of α1-subunit (PY-17) or caveolin-1 (C2-7). Together, these data support a role for cardiotonic steroid-
mediated interaction between Na\(^+\)/K\(^+\)-ATPase and CEACAM1 in the setting of chronic renal failure and insulin resistance.
3.2 Introduction

Patients with chronic renal failure (CRF) exhibit an increased incidence of cardiovascular and metabolic complications (Muntner et al., 2002; Paparello et al., 2002; Sarnak et al., 2003; Mandavia et al., 2013; Van Buren and Toto 2013). Cardiotonic steroids (CTS)—including ouabain and marinobufagenin (MBG)—are elevated during CRF (Priyadarshi et al., 2003; Komiyama et al., 2005; Kolmakova et al., 2011) and have been implicated, via their roles in regulating blood pressure and natriuresis (Periyasamy et al., 2005; Bagrov et al., 2009), in the cardiovascular pathology in these patients (de Wardener 1996; Fedorova et al., 2002, 2005). As several animal models, as with patients, with chronic renal failure also demonstrate insulin resistance and impaired glucose tolerance (Panchal and Brown 2011; Liao et al., 2012), we sought to investigate the association between renal failure and insulin resistance.

Cardiotonic steroids can bind to and signal through a pool of caveolar Na\(^+\)/K\(^+\)–ATPase in target tissues to activate various factors including Src (Liu et al., 2003, 2005). Phosphorylated Src further propagates the signal by facilitating interaction between caveolar Na\(^+\)/K\(^+\)–ATPase (Tian et al., 2006) and epidermal growth factor receptor (EGFR), which in turn recruits Shc (Haas et al., 2002; Liu et al., 2004). Najjar and colleagues have reported that Shc is crucial for interaction between insulin receptor (IR) and CarcinoEmbryonic Antigen Cell Adhesion Molecule (CEACAM) 1, and endocytosis of this complex (Li Calzi et al., 1997; Najjar et al., 1998; Poy et al., 2002a) en route to receptor recycling and insulin clearance (Choice et al., 1998). This is evidenced by the onset of insulin resistance and impaired insulin clearance in the liver of mice with
functionally inactive CEACAM1 (Poy et al., 200b; Park et al., 2006; Ghosh et al., 2010). Hepatic changes consistent with clinical diagnosis of nonalcoholic steatohepatitis (NASH) in humans were also evident in these CEACAM1-null mice when fed a high-fat diet (Ghosh et al., 2010). Moreover, CEACAM2 expressed in mice also appears to be involved in regulating metabolism and peripheral insulin action (Heinrich et al., 2010; Patel et al., 2012).

Elevated CTS may compromise glucose homeostasis through their effects on CEACAM1 protein that is most abundant in the liver, followed by the kidney (Cheung et al., 1993; Prall et al., 1996). We have previously demonstrated higher level of circulating MBG in the 5/6th partially nephrectomized rat, a well-accepted model of chronic renal failure (Kennedy et al., 2006, 2008). We postulate that marinobufagenin can signal via the sodium pump to modulate interaction with CEACAM1 and the insulin receptor, and contribute to the development of insulin resistance and chronic renal failure. To address this, we examined the effects of MBG and ouabain on CEACAM1 localization and expression in the liver and kidney using both in vivo and in vitro models. Following validation of relevant methodology in our systems, we investigated concurrent changes in sodium pump, insulin receptor and epidermal growth factor receptor expression and intracellular localization in response to these cardiotonic steroids.
3.3 Materials & Methods

Chemicals of the highest purity available were obtained from Sigma (St. Louis, MO). Monoclonal and polyclonal antibodies against Na\(^+\)/K\(^+\)–ATPase \(\alpha\)-1 subunit (clone C464.4), EGFR, and EEA1 were obtained from Upstate Biotechnology (Lake Placid, NY, USA). Antibody against caveolin-1 (clone C060) was obtained from BD Transduction Laboratories (Lexington, KY). Monoclonal antibody against clathrin heavy chain (CHC, clone x22) was obtained from Affinity BioReagents (Golden, CO). Polyclonal antibodies against IR\(\beta\), caveolin-1, cSrc, Rab7, as well as horseradish peroxidase-conjugated goat anti-mouse and goat anti-rabbit IgG were obtained from Santa Cruz Biotechnology (Santa Cruz, CA) and were used for Western blots. Monoclonal antibody against Na\(^+\)/K\(^+\)–ATPase \(\alpha\)-1 subunit (clone \(\alpha\)6F) was obtained from Developmental Studies Hybridoma Bank (University of Iowa, Iowa City, IA). Normal mouse IgG and rabbit IgG were purchased from Sigma. Optitran nitrocellulose membrane was obtained from Schleicher & Schuell (Keene, NH). EZ-Kink sulfo-NHS-ss-Biotin was obtained from Pierce Biotechnology (Rockford, IL).

Cell Culture

The pig renal proximal tubule cell line, LLC-PK1, was obtained from the American Tissue Type Culture Collection (Manassas, VA), and cultured to confluent condition as described before (Liu et al., 2002). Briefly, the cells were maintained at 37°C
in a humidified atmosphere containing 5% CO₂ in Dulbecco’s Modified Eagle’s Medium (DMEM, Sigma) with 10% (v/v) fetal bovine serum (FBS, Sigma), penicillin (100 U/mL), and streptomycin (100 µg/mL) to subconfluent conditions. Cells were passaged with 0.05% trypsin. Cell-surface protein biotinylation studies were conducted using hepatoma H4IIE cells. For experiments, cells were serum-started for 12-18 hours before treatment.

In case of experiments of immunostaining, LLC-PK1 cells were grown to confluence (approximately 6-7 days) on the 24-mm polycarbonate Transwell culture filter inserts (filter pore size 0.4 µm, Costar Co.; Cambridge, MA). Medium was replaced daily until 12 hours before experiments, at which time the cells were serum-starved as reported previously (Liu et al., 2002). LLC-PK1 cells expressing mock-vehicle (P-11, as control), Na⁺/K⁺-ATPase-α1 siRNA (PY-17, as α1-depleted cells), and caveolin-1 siRNA (C2-7, as caveolin-1 depleted cells) were cultured in the same manner as the parent LLC-PK1 cells.

**Immunofluorescence**

Cells grown to confluence on the 24-mm Transwell filters were fixed and permeabilized as described by Muth et al. (1998). Briefly, cells were fixed with cold absolute methanol, permeabilized in permeabilization buffer [PBS-Ca-Mg with 0.3% Triton X-100 and 0.1% bovine serum albumin (BSA), freshly prepared] for 15 minutes, and blocked with goat serum dilution buffer (GSDB) [20 mmol/L sodium phosphate, pH
7.4, with 150 mmol/L NaCl, 0.3% Triton X-100, and 16% (v/v) filtered normal goat or horse serum] for 30 minutes at room temperature. The cells were then probed with primary antibody for 90 minutes at room temperate or overnight at 4°C (monoclonal anti-α1 antibody, Upstate; polyclonal anti-CEACAM1 antibody from Dr. Najjar, 1:100 dilution in GSDB). After 3 washes with permeabilization buffer, the cells were incubated with Alexa Fluor® 546-conjugated anti-mouse or Alexa Fluor® 488-conjugated anti-rabbit antibody for 1 hour at room temperature. After 3 additional washes, specimens were mounted using Prolong Anti-fade medium (Molecular Probes, Eugene, OR).

All of the images were acquired via a Leica TCP SP5 broadband confocal microscope system (Leica, Mannheim, Germany) with a 63X oil-immersion objective. A series of optical sections (0.5 μm thickness) were collected, and analyzed with Leica software. The confocal microscope studies were performed using resources of the Advanced Microscopy and Imaging Center at the University of Toledo Health Science Campus.

**Preparations of Endosomes**

Endosomes were fractionated on a floating gradient using the technique of Gorvel et al. (1991). Briefly, control and treated cells were washed twice with ice-cold PBS-Ca-Mg solution, and once with ice-cold PBS. The cells were collected in PBS and centrifuged at 4°C at 3000g for 5 minutes. The pellet was resuspended in 3 mL of the homogenization buffer (250 mmol/L sucrose in 3 mmol/L imidazole, pH 7.4) and
recentrifuged at 4°C at 3000g for 10 minutes. The pellet was resuspended in 1.0 mL of homogenization buffer (with 10 μg/mL aprotinin, 10 μg/mL leupeptin, 1 mmol/L PMSF, and 0.5 mmol/L EDTA), and gently homogenized on ice using a Dounce homogenizer (15-20 strokes), followed by a centrifugation (4°C at 3000g for 10 minutes). The supernatant was adjusted to 46% sucrose using a stock solution of 62% sucrose in 3 mmol/L imidazole (pH 7.4) and loaded at the bottom of a centrifuge tube, to which was sequentially added 16% sucrose (3 mL) in 3 mmol/L imidazole and 0.5 mmol/L EDTA in ²H₂O, 10% sucrose in the same buffer (3 mL), and finally homogenization buffer (1 mL). The gradient was centrifuged at 4°C at 130,000g in a Beckmann SW 40Ti rotor for 75 minutes. Early endosomal fraction was collected at the 16%–10% sucrose interface, while the late endosomal fraction was collected at the interface between 10% sucrose and homogenization buffer. The identity of early and late endosomal fractions was determined with polyclonal antibodies raised against EEA1 and Rab7, which are early and late endosome marker proteins, respectively.

**Western Blot**

Immunoblotting was performed as described previously (Liu et al., 2004). Briefly, after treatment with different conditions, cells were washed with ice-cold PBS solution and solubilized in ice-cold lysis buffer. Cell lysates were cleared by microcentrifugation, and protein concentrations in supernatants were determined using the BioRad method. Cell lysates (50 μg/lane), endosomal fractions (15 μg/lane), or immunoprecipitates (1
mg/lane) were separated by 4-15% gradient sodium dodecyl sulfate-polyacrylamide gel
electrophoresis (SDS-PAGE; BioRad), and transferred to nitrocellulose membrane. After
transfer, membranes were blocked with 5% milk in TBS-T (Tris-HCl 10 mmol/L, NaCl
150 mmol/L, Tween 20, 0.05%; pH 8.0) solution for 1 hour at room temperate, and
immunoblotting was performed. Detection was performed with the Enhanced
Chemiluminescence Plus Western Blotting Detection System (Amersham,
Buchinghamshire, United Kingdom). Multiple exposures were analyzed to assure that the
signals were within the linear range of the film. Autoradiograms were scanned with a
Bio-Rad GS-670 imaging densitometer (BioRad, Herculese, CA) to quantify signals (Liu
et al., 2002).

Animals

Male, Dawley rats subjected to either sham surgery without partial nephrectomy
(SS; n=18) or partial nephrectomy (PNx; n=20) were used for all studies. Experimental
renal failure was induced by 5/6th nephrectomy, namely removal of the right kidney and
selective infarction of 2/3rd of the left kidney with silk ligatures, as described previously
(Kennedy et al., 2003). All procedures were approved by the Institutional Animal Care
and Utilization Committee. Measurement of MBG in plasma was determined at four
weeks post surgery following extraction with C-18 columns as described previously
(Priyadarshi et al., 2003)
**Statistical Analysis**

Data are presented as the mean ± standard error of mean (SEM). Data obtained were first tested for normality and then subjected to parametric analysis. For comparison of more than two groups, one-way ANOVA was employed using the Student *t*-test with Bonferroni’s correction for multiple comparisons for post-hoc analysis (Wallenstein et al., 1980). Statistical analysis was performed with SPSS software.
3.4 Results

Hepatic CEACAM1 Expression Reduced in Partially Nephrectomized Rats.

As marinobufagenin is elevated during chronic renal failure, we first confirmed this in our 5/6\textsuperscript{th} partially nephrectomized male Dowley rats. Indeed, circulating plasma MBG levels were significantly higher in rats with partial nephrectomy (PNx) as compared to control (SS; Figure 1A). We next examined CEACAM1 protein expression in the liver, where it is abundantly expressed, of partially nephrectomized rats using Western blot analysis. Hepatic CEACAM1 protein expression was attenuated by approximately 50\% in partially nephrectomized rats (Figures 1B and 1C). This decrease in CEACAM1 protein expression was not accompanied by a detectable decrease in Ceacam1 mRNA expression (data not shown).

Reduced MBG- and Insulin-Induced Cell Surface Expression of Na\textsuperscript{+}/K\textsuperscript{+}-ATPase -\(\alpha\)1 subunit, CEACAM1 and IR\(\beta\) in Hepatoma H4IIE Cells.

We investigated whether marinobufagenin and/or insulin exert an effect on the cell-surface expressions of Na\textsuperscript{+}/K\textsuperscript{+}-ATPase, CEACAM1, or IR in the liver. Consistent with previous findings in kidney LLC-PK1 cells, MBG treatment (10 nM, 30 minutes) did not affect the total protein expression of Na\textsuperscript{+}/K\textsuperscript{+}-ATPase-\(\alpha\)1 subunit in rat hepatoma H4IIE cells (data not shown). Subsequently, MBG (10 nM, 30 minutes), with or without
insulin (100 nM, 30 minutes), treatments of H4IIE liver cells markedly reduced cell-surface expression of the sodium pump α1 subunit as assayed by cell-surface protein biotinylation (Figure 2A). Moreover, both CEACAM1 and IRβ expressions were also diminished on the surface of hepatic H4IIE cells in response to exogenous MBG or insulin treatment. Similar to the effect on α1 subunit, concurrent treatment with MBG and insulin did not produce synergistic endocytotic effects on CEACAM1 (Figures 2B and 2C) or IRβ (Figures 2D and 2E) in these hepatoma H4IIE cells. No statistical analysis was performed due to insufficient replications (n=3) of these experiments.

**Anti-Rat CEACAM1 Antibody can Detect CEACAM1 in Porcine Renal Proximal Tubule LLC-PK1 Cells.**

Since the kidney is another important target of insulin action, we utilized the porcine renal proximal tubule LLC-PK1 cell line as an *in vitro* model for further mechanistic studies. CEACAM1 protein expression in these kidney cells was determined using the above mentioned anti-rat CEACAM1 antibody with Western Blot analysis. CEACAM1 protein was detectable, though expressed at a lower level, in porcine LLC-PK1 cells as compared to the rat liver H4IIE cells where it known to be abundantly expressed (Figure 3). Interestingly, SYF cells deficient in Src also expressed CEACAM1 protein (data not shown).
Ouabain- and Insulin-Induced Translocation of CEACAM1 From the Plasma Membrane to Intracellular Region of Renal Proximal Tubule LLC-PK1 Cells.

Immunofluorescence studies were conducted next to determine cellular localization of CEACAM1 in the kidney. Initial confocal studies demonstrated that CEACAM1 (green fluorescence) and Na\(^+\)/K\(^+\)–ATPase \(\alpha\) subunit (red fluorescence) co-localized to the plasmalemma of renal proximal tubule LLC-PK1 cells under basal conditions. In particular, confocal X-Z plane images show that both \(\alpha\) subunit and CEACAM1 expressions were greater on the basolateral as compared to the apical aspect of these polarized renal proximal tubule LLC-PK1 cells (Figure 4F, bottom row). Exposure to ouabain (100 mM, 0 to 12 hours) produced a temporal intracellular redistribution of CEACAM1 and sodium pump \(\alpha\)-subunit to the intracellular region of LLC-PK1 cell derivatives (P11–control; PY-17–\(\alpha\) knock-down; and C2-7–caveolin-1 knock-down), with marked internalization as early as 15 minutes (Figure 4A and 4B). Ouabain treatment for 30 minutes also showed significant internalization and was used in all subsequent experiments (Gupta et al., 2012). In comparison, ouabain-induced CEACAM1 protein internalization was not as marked in PY-17 or C2-7 cells as compared to control LLC-PK1 cells (Figures 5 and 6).
3.5 Discussion

Using a well studied experimental 5/6th partial nephrectomy rat model of chronic renal failure, we found reduced CEACAM1 protein in the liver, a major site of insulin clearance as demonstrated in transgenic L-SACC mice over-expressing functionally inactive hepatic CEACAM1 (Najjar 2002; Dai et al., 2004). This attenuated CEACAM1 protein, without a change in mRNA, level suggests modulation to occur at the post-transcriptional or -translational level. Subsequent studies addressed whether these potential CTS-mediated effects are via post-translational modification. Cardiotonic steroids signal through the Na⁺/K⁺-ATPase by binding to its α1 subunit and inducing pump endocytosis in renal LLC-PK1 cells (Liu et al., 2005). We investigated whether MBG has a similar effect on Na⁺/K⁺-ATPase-α1 subunit in the liver, where the sodium pump is also expressed (Yagawa et al., 1990). We report a decline in insulin-stimulated cell-surface α1 expression in H4IIE cells, suggesting similarly enhanced endocytotic effect of insulin in the liver. Moreover, increased internalization of cell-surface proteins including hepatic CEACAM1 and IRβ, as well as sodium pump, in response to cardiotonic steroids and insulin suggests a common signaling mechanism that may contribute to insulin resistance observed in chronic renal failure patients. Intracellular redistribution of these molecules without a decline in synthesis warrants these CTS-associated responses, at least in part, due to post-translational modification.

Our data of lower CEACAM1 protein in the rat kidney, as compared to liver, is consistent with previously reported decreased renal Ceacam1 mRNA levels in mice (Han
et al., 2001). The sharper band pattern of CEACAM1 in the kidney versus a broader band detected in the liver could reflect distinct tissue-specific protein glycosylation. These and other experiments presented in Appendix A validated continued use of material and methods for our in vivo and in vitro studies in the kidney.

The sodium pump is expressed on basolateral aspect of polarized renal epithelial cells (Liu et al., 2004) While CEACAM1-S and CEACAM1-L are present homogenously on cell surface of non-polarized hepatocytes, Prall reported CEACAM1-L to be exclusively expressed on the apical aspect of polarized epithelial cells (Prall et al., 1996). In contrast, more recent studies have localized the protein to apical, and more abundantly on basolateral aspects of polarized renal epithelial MDCK cells (Sundberg et al., 2004). Consistent with these reports, CEACAM1 protein was abundantly more expressed on the basolateral aspect of polarized renal LLC-PK1 cells in our immunostaining studies, where it can respond to CTS-mediated signaling through the basolateral sodium pump. In studies with targeted mutation of CEACAM1-L, Sundberg showed that phosphorylation of Tyr515 on the long cytoplasmic domain of CEACAM1-L is required for its localization to the lateral aspect of polarized renal MDCK cells (Sundberg et al., 2004). Interestingly, though it is not phosphorylated by insulin receptor tyrosine kinase, intact Tyr513 in mice is also required for phosphorylated CEACAM1 to activate serine kinase in response to insulin (Najjar et al., 1995). Taken together, the Tyr513 residue, which is important in intracellular trafficking and insulin response, could also be a target for ouabain-induced signaling through the sodium pump.

As expected, sodium pump α1 staining was markedly reduced in PY-17 cells, which express approximately 8% Na+/K+-ATPase-α1 expression and 21% of ouabain-
responsive ATPase activity, as compared to control P11 cells (Liang et al., 2007). Xie also reported decreased sodium pump ATPase activity in C2-7 cells with reduced caveolin-1 expression. As shown in Appendix B, Src phosphorylation was impaired in C2-7 cells lacking caveolin, with detectable pSrc in control P11 cells (Figure B-2). Ouabain did not stimulate intracellular CEACAM1 localization without sufficient sodium pump “singalosomes” in these PY-17 cells. Similarly, caveolin-1 is also needed for CTS-induced CEACAM1 translocation, as evident by attenuated intracellular CEACAM1 localization in response to ouabain in caveolin-1 deficient C2-7 cells. Caveolin-1 protein was undetectable in early endosomes of C2-7 cells, with intact expression in P11 cells (Appendix B, Figure B-1). Elsewhere, we have demonstrated that caveolin-1 was also required for early endosomal accumulation of CEACAM1 in response to ouabain, but not insulin (Gupta et al., 2012). Together, these data indicate that Na+/K+-ATPase and caveolin-1 play a role in CTS-mediated CEACAM1 internalization from the plasma membrane of renal proximal tubule cells. Insulin-stimulated CEACAM1 accumulation in early endosomes is likely due to the intact clathrin-mediated internalization in these cells. Interestingly, as Src is important in facilitating sodium pump signaling, the presence of CEACAM1 in Src-deficient mouse embryo SYF cells also renders this cell line as an important tool in deciphering the role of Src in CTS-mediated signaling through the sodium pump.

Taken together, our data suggest that cardiotonic steroids and insulin share a common signaling mechanism for Na+/K+-ATPase, CEACAM1, and IR internalization and signaling in the kidney. These findings extend our knowledge of a shared signaling mechanism between CTS and insulin on key players involved in insulin regulation.
Further research in this area could contribute significantly to a better mechanistic understanding of the development of insulin resistance in chronic renal failure, as well as improved therapeutic outcomes for these patients.

3.6 Acknowledgments

Some of these data were presented in abstract form at the 2004 Scientific Session of the American Society of Nephrology. We acknowledge Dr. David Kenney and Dr. Jiang Liu for their expert scientific input and advice, and Mats Fernstrom for his technical assistance.
3.7 References


Komiyama Y, Dong XH, Nishimura N, Masaki H, Yoshika M, Masuda M, Takahashi H.


3.8 Figure Legends

**Figure 3-1.** Plasma MBG levels and CEACAM1 protein expression in liver tissue of rats subjected to sham surgery (SS, n=4) or partial nephrectomy (PNx, n=6). A–Plasma MBG levels. B–Autoradiograph of hepatic CEACAM. C–Relative Optical Density. SS–Sham Surgery. Student’s t-test, *p<0.05.

**Figure 3-2.** Reduced cell-surface expression of Na⁺/K⁺–ATPase-α1 (A), CEACAM1 (B-C) and IRβ (D-E) with MBG+Insulin in liver H4IIE cells. C–control; I–insulin (100 nmol/L, 30 minutes); M–marinobufagenin (10 nmol/L, 30 minutes). N=3 from each group; no statistical analysis performed.

**Figure 3-3.** Relative expression of total CEACAM1 protein in rat liver H4IIE (50 micrograms; *Lane 1*) and porcine kidney LLC-PK1 (50 or 100 micrograms; *Lanes 2-3*) cells.

**Figure 3-4.** Temporal distribution of CEACAM1 (green fluorescence) and Na⁺/K⁺–ATPase-α1 subunit (red fluorescence) in renal proximal LLC-PK1 cells without (A) or treated with ouabain (100 nmol/L) for 15 minutes (B), 30 minutes (C), 60 minutes (D), or 12 hours (E). Merged image shows colocalization (yellow fluorescence). Confocal images are in the X-Y plane (A through E) or X-Z plane (F). N=5 samples studied from each group.
Figure 3-5. Cellular distribution of CEACAM1 (A) (green fluorescence) and Na⁺/K⁺--ATPase-α1 subunit (B) (red fluorescence) expression in α1–knock-down PY-17 cells treated without or with ouabain (100 nmol/L; 30 minutes). Merged image shows colocalization (yellow fluorescence). N=5 samples studied from each group.

Figure 3-6. Cellular distribution of CEACAM1 (A) (green fluorescence) and Na⁺/K⁺--ATPase-α1 subunit (B) (red fluorescence) expression in caveolin-1–knock-down C2-7 cells treated without or with ouabain (100 nmol/L; 30 minutes). Merged image shows colocalization (yellow fluorescence). N=5 samples studied from each group.
3.9 Figures

Figure 3-1.
Figure 3-2.
Figure 3-3.
Figure 3-4.
Figure 3-5.
Figure 3-6.
Chapter 4

Manuscript

Gupta et al., Hypertension 2012; 59:665-672.

OUABAIN AND INSULIN INDUCE SODIUM PUMP ENDOCYTOSIS IN RENAL EPITHELIUM

by

Shalini Gupta¹, Yanling Yan¹, Deepak Malhotra¹, Jiang Liu¹,
Zijian Xie¹,², Sonia M Najjar²,³ and Joseph I Shapiro¹,².

From

The Departments of Medicine¹ and of Physiology and Pharmacology², and the Center for Diabetes and Endocrine Research³. University of Toledo College of Medicine, Toledo, Ohio, 43614
Address correspondence to:

Joseph I. Shapiro, MD, Mail Stop #1186 Health Science Campus, University of Toledo College of Medicine, 3000 Arlington Avenue, Toledo, Ohio 43614-2598.

Phone: 419-383-6030;

FAX: (419)383-6244;

E-mail: Joseph.Shapiro@utoledo.edu

Short title: Ouabain and Insulin signaling in LLC-PK1 cells

Key words: chronic renal insufficiency; renal proximal tubule cell; endocytosis
4.1 Abstract

Cardiotonic steroids signaling through the basolateral sodium pump (Na/K-ATPase) have been shown to alter renal salt handling in intact animals. Because the relationship between renal salt handling and blood pressure is a key determinant of hypertension, and patients with insulin resistance are frequently hypertensive, we chose to examine whether there might be competition for resources necessary for receptor-mediated endocytosis.

In LLC-PK1 cells, the Na/K-ATPase-α1 and carcinoembryonic antigen cell adhesion molecule 1, a plasma membrane protein that promotes receptor-mediated endocytosis, colocalized in the plasma membranes and translocated to the intracellular region in response to ouabain. Either ouabain or insulin alone caused accumulation of carcinoembryonic antigen cell adhesion molecule, as well as insulin receptor-β, and epidermal growth factor receptor in early endosomes, but no synergy was demonstrable. Like ouabain, insulin also caused c-Src activation. When caveolin or Na/K-ATPase α1 expression was knocked down with small interfering RNA, insulin but not ouabain induced carcinoembryonic antigen cell adhesion molecule 1, insulin receptor-β, and epidermal growth factor receptor endocytosis.

To determine whether this might be relevant to salt handling in vivo, we examined salt loading in mice with null renal carcinoembryonic antigen cell adhesion molecule 2 expression. The null renal carcinoembryonic antigen cell adhesion molecule 2 animals demonstrated greater increases in blood pressure with increases in dietary salt than control animals.
These data demonstrate that cardiotonic steroids and insulin compete for cellular endocytosis resources and suggest that, under conditions where circulating insulin concentrations are high, cardiotonic steroid mediated natriuresis could be impaired.
4.2 Introduction

Insulin binding induces phosphorylation of the insulin receptor (IR), carcinoembryonic antigen-related cell adhesion molecule 1 (CEACAM1), and its substrates to activate downstream signaling pathways. Phosphorylation of CEACAM1, a surface membrane glycoprotein, promotes insulin endocytosis via its receptor, followed by its degradation. In agreement with receptor-mediated insulin uptake and degradation constituting the basic mechanism of its clearance in liver and kidney, null mutation of CEACAM1 impairs insulin clearance to cause hyperinsulinemia and insulin resistance.²,³

We have shown previously that cardiotonic steroids induce endocytosis of the plasmalemmal Na/K-ATPase in renal proximal tubule cells to increase urinary sodium excretion.⁴ Researchers have recently shown recently that, although some cardiotonic steroids may promote hypertension, loss of their natriuretic effects may actually exacerbate salt-dependent hypertension.⁵-⁷

Similar to IR, the epidermal growth factor receptor (EGFR) also phosphorylates CEACAM1, an event that, in turn, mediates their complex formation and regulation of post-receptor signaling.⁸ Given that EGFR is transactivated by Na/K-ATPase–initiated signaling, the current study tests whether CEACAM1 is also involved in this process.
4.3 Materials and Methods

Chemicals and Antibodies

Chemicals of the highest purity available were obtained from Sigma (St. Louis, MO). Monoclonal and polyclonal antibodies against Na/K-ATPase-α−1 subunit (clone C464.4), EGFR, and early endosomal antigen 1 were obtained from Upstate Biotechnology (Lake Placid, NY). Antibody against caveolin-1 (clone C060) was obtained from BD Transduction Laboratories (Lexington, KY). Monoclonal antibody against clathrin heavy chain (clone x22) was obtained from Affinity BioReagents (Golden, CO). Polyclonal antibodies against IR-β subunit, caveolin-1, c-Src, and Rab-7, as well as horseradish peroxidase–conjugated goat antimouse and goat antirabbit IgG, were obtained from Santa Cruz Biotechnology (Santa Cruz, CA) and were used for Western blots. Monoclonal antibody against the IR-β subunit was obtained from AnaSpec (Freemont, CA). Monoclonal antibody against the Na/K-ATPase-α-1 subunit (clone α6F) was obtained from Developmental Studies Hybridoma Bank (University of Iowa, Iowa City, IA). Normal mouse IgG and rabbit IgG were purchased from Sigma. Optitran nitrocellulose membrane was obtained from Schleicher & Schuell (Keene, NH).
Cell Culture

The pig renal proximal tubule cell line, LLC-PK1, was obtained from the American Tissue Type Culture Collection (Manassas, VA) and cultured to confluent condition as described before. In immunostaining, LLC-PK1 cells were grown to confluence on the 24-mm polycarbonate Transwell culture filter inserts (filter pore size 0.4 μm; Costar Co., Cambridge, MA), as also reported previously. LLC-PK1 cells expressing mock-vehicle (P-11, as control), Na/K-ATPase–α1 small interfering RNA (PY-17, as α1-knock down cells), and caveolin-1 small interfering RNA (C2-7, as caveolin-1 depleted cells) were cultured in the same manner as the parent LLC-PK1 cells.

Immunofluorescence

Cells grown to confluence on the 24-mm Transwell filters were fixed and permeabilized as described by Muth et al. The cells were then probed with primary antibody for 90 minutes at room temperate or overnight at 4°C (monoclonal anti-α1 antibody, Upstate; polyclonal anti-CEACAM1 antibody, 1:100 dilution in goat serum dilution buffer). After 3 washes with permeabilization buffer, the cells were incubated with Alexa Fluor 546-conjugated or Alexa Fluor 488-conjugated secondary antibody for
1 hour at room temperature. After 3 additional washes, specimens were mounted using Prolong Anti-fade medium (Molecular Probes, Eugene, OR).

All of the images were acquired via a Leica TCP SP5 broadband confocal microscope system (Leica, Mannheim, Germany) with a 63X oil-immersion objective and analyzed with Leica software. The confocal microscope studies were performed using resources of the Advanced Microscopy and Imaging Center at the University of Toledo Health Science Campus.

**Immunoprecipitation**

Immunoprecipitation experiments were performed as described by Chibalin et al and published previously from our laboratory. LLC-PK1 cells were lysed in ice-cold radioimmunoprecipitation assay lysis buffer. Insoluble material was removed by centrifugation (14,000 X 10 minutes at 4°C). Aliquots of supernatant (total of 1 mg protein) were immunoprecipitated overnight at 4°C with monoclonal anti-Na/K ATPase-α1 subunit antibody (Upstate). Immunoprecipitates were incubated with protein G-agarose beads, rotating for 2 hours at 4°C. Beads were washed five times in radioimmunoprecipitation assay lysis buffer, and immunoprecipitated proteins were eluted with Laemmli sample buffer and analyzed by Western blot.
Preparations of Endosomes

Endosomes were fractionated on a floating gradient using the technique of Gorvel et al.\textsuperscript{14} The early endosomal fraction was collected at the 16% to 10% sucrose interface. The identity of early endosomal fractions was determined with antibodies against early endosome protein marker early endosomal antigen 1, as we have described previously.\textsuperscript{12, 13}

Western Blot

Immunoblotting was performed as described previously.\textsuperscript{12} Briefly, cell lysates (50 µg per lane), endosomal fractions (15 µg per lane), or immunoprecipitates (from 1 mg total protein per sample) were separated by 4% to 15% gradient SDS-PAGE (BioRad) and transferred to nitrocellulose membrane. After transfer, membranes were blocked with 5% milk in Tris-buffered saline-Tween (Tris-HCl 10 mmol/L, NaCl 150 mmol/L, Tween 20, 0.05%; pH 8.0) for 1 hour at room temperate, and immunoblotting was performed. Detection was performed with the enhanced chemiluminescence Plus Western Blotting Detection System (Amersham, Buchinghamshire, United Kingdom). Multiple exposures were analyzed to assure that the signals were within the linear range of the film. Autoradiograms were scanned with a Bio-Rad GS-670 imaging densitometer (BioRad, Herculese, CA) to quantify signals.\textsuperscript{9}
Animals

The generation of Ceacam2-null (Ce2<sup>−/−</sup>) mice was described previously. Male mice from 4 to 8 months of age were studied. Animals were kept in a 12-hour dark/light cycle and fed standard regular chow and tap water ad libitum. All procedures were approved by the Institutional Animal Care and Utilization Committee.

Statistical Analysis

Data are presented as the mean ± SEM. Data obtained were first tested for normality and then subjected to parametric analysis. For comparison of more than two groups, 1-way ANOVA was used using the Student t test with Bonferroni’s correction for multiple comparisons for post hoc analysis. Statistical analysis was performed with SPSS software.
4.4 Results

Na/K-ATPase–α1 and CEACAM1 Co-Localize to the Plasmalemma of Porcine Renal Proximal Tubule LLC-PK1 Cells

To determine whether CEACAM1 protein is expressed in porcine renal proximal tubule LLC-PK1 cells, an *in vitro* model extensively utilized in our laboratory, we initially carried out Western analysis of whole cell lysates. CEACAM1 protein was easily detected in LLC-PK1 cells, albeit to a much lower extent than rat hepatoma H4IIE cells (data not shown). Subsequently, immunofluorescence analysis demonstrated that CEACAM1 was mostly localized to the plasmalemma of monolayer LLC-PK1 cells grown on Transwell membranes (Figure 1, top row, green fluorescence).

This expression pattern of CEACAM1 was comparable to that of Na/K-ATPase-α1 subunit (Figure 1, top row, red fluorescence), as we and others have previously reported. Furthermore, both of these proteins colocalized, as revealed by merging analysis (Figure 1, top row, yellow fluorescence).
Ouabain- and Insulin-Induced Accumulation of CEACAM1 in Early Endosomal Fractions of LLC-PK1 Cells

Ouabain (100 nM, 30 minutes) increased intracellular content of Na/K-ATPase-\(\alpha_1\) subunit in LLC-PK1 cells (Figure 1, bottom row, red fluorescence). Intracellular CEACAM1 content also increased in response to the same ouabain treatment (Figure 1, bottom versus top row, green fluorescence). These data suggested that the sodium pump \(\alpha_1\) subunit and CEACAM1 are coexpressed on the plasmalemma of LLC-PK1 cells, and both undergo internalization in response to ouabain.

Western blot analysis revealed the presence of CEACAM1 in the early endosomal fractions of LLC-PK1 cells (Figure 2A). In comparison with control cells, ouabain treatment (100 nM, 30 minutes) causes increases in early endosomal content of CEACAM1 by \(~2.5\)-fold (Figure 2A and 2B), IR-\(\beta\) subunit by \(~3\)-fold (Figure 2A and 2C), and EGFR by \(~1.5\)-fold (Figure 2A and 2D). Ouabain also increased the early endosomal content of the \(\alpha_1\) subunit of the Na/K-ATPase, as we have previously reported\(^{12}\) (data not shown).

We next investigated whether insulin exerts a synergistic effect with ouabain on CEACAM1 internalization and whether CEACAM1 internalization depends on Na/K-ATPase signaling. To address this question, we utilized LLC-PK1 cells stably transfected with mock-expressing vector alone (P11), or with small interfering RNA expressing vectors of knockdown Na/K-ATPase \(\alpha_1\)-subunit expression (PY-17) or knockdown
Like ouabain, insulin (100 nM, 30 minutes) induced early endosomal accumulation of CEACAM1, IR-\(\beta\), and EGFR proteins in P11 control cells (Figure 3A and 3B). However, concurrent treatment with ouabain and insulin did not produce synergistic endocytic effects in P11 cells (Figure 3A and 3B).

In the PY-17 and C2-7 cells where we have previously shown ouabain signaling through the Na/K-ATPase to be minimal,\(^{19}\) insulin, but not ouabain, induced the endosomal accumulation of these receptors and CEACAM1 (Figure 3C through 3F). Moreover, ouabain failed to influence the effect of insulin on the internalization of these proteins in both PY-17 and C2-7 cells.

**Na/K-ATPase-\(\alpha1\) and Insulin Receptor \(\beta\) Co-Localize to the Plasmalemma of Porcine Renal Proximal Tubule LLC-PK1 Cells**

Immunofluorescence analysis demonstrated that sodium pump-\(\alpha1\) and IR-\(\beta\) subunits were localized to the plasmalemma of monolayer LLC-PK1 cells grown on Transwell membranes. Ouabain increased internalization of \(\alpha1\) subunit as before, and insulin treatment, with or without ouabain, also caused internalization of both \(\alpha1\) and IR-\(\beta\) subunits (Figure 4).
Ouabain- and Insulin-Induced Accumulation of Sodium Pump-α1 subunits in Early Endosomal Fractions of LLC-PK1 Cells

Consistent with our previous reports, ouabain increased sodium pump-α1 subunit protein accumulation in early endosomal fraction of LLC-PK1 cells. Interestingly, insulin alone also increased early endosomal α1 subunit accumulation, but concurrent ouabain and insulin treatment was neither synergistic nor additive (Figure 5A and 5B).

Ouabain- and Insulin-Induced c-Src Phosphorylation in LLC-PK1 Cells

As above, LLC-PK1 cells were treated with ouabain and/or insulin for 30 minutes, and c-Src phosphorylation was determined. Relative to control, ouabain stimulated c-Src phosphorylation in whole-cell lysates by ~2.5-fold (Figure 6A and 6B), as well as in early endosomal fractionations (Figure 6C and 6D). Interestingly, insulin induced a comparable effect to that of ouabain on c-Src phosphorylation in early endosomal fractions (Figure 6C and 6D). Concomitant treatment with insulin and ouabain produced no synergistic induction on c-Src phosphorylation in either whole-cell lysates (data not shown) or early endosomal fraction (Figure 6C and 6D).
The Effect of Ouabain and Insulin on Interaction Amongst Na/K-ATPase-α1, CEACAM1, and Caveolin-1 in LLC-PK1 Cells

To elucidate whether there is an interaction between α1 subunit and CEACAM1, LLC-PK1 cells were treated with ouabain and/or insulin for 1 hour and immunoprecipitated with anti-Na/K-ATPase-α1 subunit antibody. Immunoblotting with antibodies against CEACAM1 and α1 (as control) revealed that ouabain treatment enhanced the interaction between CEACAM1 and the α1 subunit (Figure 7A and 7B). Insulin similarly augmented α1–CEACAM1 coprecipitation, although this did not reach statistical significance (Figure 7A and 7B). Concurrent treatment with insulin and ouabain induced a mild statistically significant increase in α1–CEACAM1 coprecipitation (Figure 7A and 7B). Immunoblotting with an antibody against caveolin-1 revealed that ouabain and insulin increased the coimmunoprecipitation of Na/K-ATPase-α1 subunit and caveolin-1 and that this effect of insulin was not synergistic with ouabain (Figure 7A and 7C).
Salt Increased Systolic Blood Pressure in Mice with Null Mutation of Ceacam2 (Cc2−/−)

CEACAM2, a highly homologous related protein to CEACAM1, is the predominant protein in murine kidney. With CEACAM proteins being expressed in kidney proximal tubules, we evaluated the role of CEACAM2 in regulating blood pressure following salt loading. Aged matched wild-type (WT) and Cc2−/− mice were switched from a control (low salt: 0.4% salt) diet to increased amounts of dietary salt (2%, 4%, or 8% NaCl). There were no differences in body weight, water consumed, and urinary volume between WT and Cc2−/− mice regardless of their dietary salt content (Table). However, systolic blood pressures measured on days 0, 2, 7, and 14 were relatively constant in WT mice when fed 2% and 4% high-salt diets and trended to increase by day 2 on 8% high-salt diet (Figure 8). In comparison, Cc2−/− mice responded with increased blood pressure almost immediately (by day 2) even on the 2% salt diet and underwent a progressive increase in response to higher salt concentration in diet (Figure 8). On 8% salt-diet, systolic blood pressure reached a maximal blood pressure as early as day 2 and remained maximally elevated during the remainder of the study in these Cc2−/− mice (Figure 8). Both WT and Cc2−/− mice responded with increased urinary sodium excretion with all of the high-salt diets in a dose dependent manner. However, no differences in the change in body weight or urinary sodium excretion were evident between the WT and Cc2−/− animals when placed on any experimental diet. The data for
the 4% NaCl diet (which was the first experimental high-salt diet the animals were exposed to) are shown in Figure 9.
4.5 Discussion

It is well established that ouabain binds to the Na/K-ATPase and induces its endocytosis via a signaling cascade, whereas binding of insulin to its receptor induces association of the receptor with CEACAM1 and internalization for ligand degradation and IR recycling.\(^1\),\(^{21,22}\)

CEACAM1 is also present on the plasmalemma in various types of cells, where it can interact with the IR via Shc adaptor protein.\(^8\),\(^{23}\) Our data suggest that both the sodium pump and CEACAM1 are capable of responding to cardiotonic steroids in the renal proximal tubule, a crucial site of blood pressure regulation and insulin clearance. It has been shown that the IR and CEACAM1 are expressed on both apical and basolateral aspects of polarized Madine-Darby canine kidney cells, although predominantly on the basolateral surface.\(^{24-26}\) Ouabain treatment of LLC-PK1 cells and a high-salt diet result in the coordinated redistribution of the apical sodium proton antiporter NHE3 through mechanisms that are yet to be elucidated,\(^{27,28}\) as well as decreases in NHE3 expression on a transcriptional level.\(^{29}\) It is the redistribution of the NHE3 which is felt to be rate limiting in terms of proximal tubular sodium handling and natriuresis, and our previous data suggest that this is a consequence of Na/K–ATPase signaling through Src.\(^{27,29}\) Our current findings suggest that the sodium pump and insulin receptor compete for resources necessary for endocytosis.

Based on our observation of ouabain-induced accumulation of CEACAM1 protein in early endosomes of LLC-PK1 cells, we performed additional studies in LLC-
PK1 cells modified to attenuate ouabain mediated endocytosis. Specifically, LLC-PK1 cell lines with siRNA down-regulation of Na/K–ATPase α1-subunit expression (PY-17 cells), caveolin-1 expression (C2-7 cells), or control small interfering RNA (P11 cells) have been developed in our laboratories. Firstly, insulin stimulated CEACAM1, EGFR, and IRβ accumulation in early endosomal fractions of P11 cells as those observed in LLC-PK1 cells. Liu et al. previously demonstrated ouabain-induced internalization of sodium pump in early endosomal fractions of LLC-PK1 cells. Here, we report that ouabain can additionally stimulate CEACAM1, EGFR, and IRβ accumulation in early endosomal fractions of P11 cells, as observed in LLC-PK1 cells. Consistent with previously published data, ouabain stimulated c-Src phosphorylation in LLC-PK1 whole cell lysates, as well as early endosomal fractions. While ouabain has been shown to induce c-Src phosphorylation in LLC-PK1 cells, effects of insulin in this system were not known to us. Our current finding of c-Src phosphorylation resulting from insulin treatment reveals an overlap in signal transduction and strengthens the possibility of cross-talk between insulin and ouabain signaling. However, a lack of synergistic or even additive effects with respect to endocytosis or c-Src activation with ouabain and insulin suggest competing pathways in the LLC-PK1 cells specifically and possibly renal proximal tubular cells in general. It is, therefore, possible that the competition for endocytosis extends to specific signaling processes. However, more studies will be necessary to test this hypothesis.

As the sodium pump interacts with c-Src to signal, we examined the effects of ouabain and insulin in cells markedly deficient in Na/K–ATPase (PY-17 cells). These cells express only 10% of total sodium pumps and have negligible ouabain-induced
Na/K–ATPase signaling activity, including c-Src activation and downstream protein kinase cascade vs. control P11 cells.\textsuperscript{18, 30} We found that without sodium pump signaling, ouabain did not stimulate CEACAM1, EGFR or IRβ accumulation in early endosomal fractions. Insulin, in the presence or absence of ouabain, still caused CEACAM1, EGFR or IRβ accumulation in early endosomal fractions. Based on these data, the signaling sodium pump is crucial for ouabain-induced endocytosis of these previously established mediators of insulin clearance. Immunoprecipitation studies confirmed the interactions between the Na/K–ATPase, CEACAM and the insulin receptor.

To assess whether this interaction of the Na/K–ATPase with CEACAM has a physiological impact, we measured systolic blood pressure in Ceacam2\textsuperscript{-/-} mice fed a diet higher in salt content (2%, 4%, or 8%) vs. control (0.4%) for two weeks. Cc2\textsuperscript{-/-} mice were more susceptible to hypertension during salt-loading than wild-type mice. Moreover, the salt content in the diet correlated with both the rapidity of onset and severity of systolic blood pressure increase. These data suggests that the renal CEACAM molecule in mice (CEACAM2) is involved in cardiotonic steroid induced Na/K-ATPase endocytosis and hence, contributes to blood pressure regulation. Interestingly, we have recently reported that in the Dahl salt sensitive strain of rat, impaired proximal tubular Na/K-ATPase endocytosis in response to salt loading in vivo or ouabain exposure in vitro can be demonstrated in comparison to the Dahl salt resistant strain. In other words, the lack of renal natriuretic response to CTS corresponded to the increases in blood pressure seen in the Dahl salt sensitive strain with salt loading\textsuperscript{33}. These data are, of course, consistent with the observed hypertension seen in the Cc2\textsuperscript{-/-} mice subjected to a high salt diet. However, additional studies are clearly required to further characterize abnormalities in insulin, or
cardiotonic steroid-induced signaling through the Na/K-ATPase “signalosome” in the development of salt-sensitive hypertension in these Cc2\(^{−/−}\) mice.

Insulin has been shown to initiate phosphorylation of the sodium pump in skeletal muscle\(^{34}\). Here, we report that insulin can stimulate \(\alpha_1\) subunit internalization in the kidney. The current studies demonstrate that ouabain-induced signaling through the sodium pump also interacts with insulin receptor-associated molecules, including CEACAM, in the kidney. Although further studies are required, the data identify a competition between ouabain and insulin mediated endocytosis and suggests a potential role for this competition in the regulation of salt excretion and, therefore, the pathogenesis and possibly treatment of hypertension seen in hyperinsulinemic states.

4.6 Perspectives

The current studies demonstrate that ouabain-induced signaling through the sodium pump also interacts with IR-associated molecules, including CEACAM, in the kidney. Although further studies are required, the data identify a competition between ouabain- and insulin-mediated endocytosis and suggest a potential role for this competition in the regulation of salt excretion and, therefore, the pathogenesis and possibly treatment of hypertension seen in hyperinsulinemic states.
4.7 Acknowledgements

We thank Carol Woods for her excellent secretarial assistance. We also acknowledge Mats A. Fernstrom and Jennifer Kalisz for their technical assistance.

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Disclosures

None.
4.8 References


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<table>
<thead>
<tr>
<th>Variable</th>
<th>0.4% Salt Diet, Day 8</th>
<th>2.0% Salt Diet, Day 8</th>
<th>4.0% Salt Diet, Day 8</th>
<th>8.0% Salt Diet, Day 8</th>
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</thead>
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<tr>
<td></td>
<td>WT</td>
<td>Cc2^{−/−}</td>
<td>WT</td>
<td>Cc2^{−/−}</td>
</tr>
<tr>
<td>Body weight, g</td>
<td>27.24±0.76</td>
<td>28.51±0.91</td>
<td>28.84±1.04</td>
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<tr>
<td>Urine output, mL/24 h</td>
<td>0.9±0.1</td>
<td>0.6±0.2</td>
<td>2.6±2.0</td>
<td>3.1±0.8</td>
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<tr>
<td>UNaV, μeq/24 h</td>
<td>53±5</td>
<td>49±7</td>
<td>405±82</td>
<td>477±92</td>
</tr>
</tbody>
</table>

Data are shown as the mean±SEM of measurements performed on 5 animals in each group. WT indicates wild-type; Cc2^{−/−}, Ceacam2-null; UNaV, urinary sodium excretion.
4.10 Figure Legends

**Figure 4-1.** Representative images showing the cellular redistribution of carcinoembryonic antigen-related cell adhesion molecule (CEACAM) 1 (green fluorescence) and Na/K-ATPase-α1 subunit (red fluorescence) after ouabain (100 nmol/L, 30 minutes) treatment in renal proximal tubule LLC-PK1 cells. Merged image shows colocalization (yellow fluorescence). N=5 samples studied from each group.

**Figure 4-2.** A, Autoradiographs of representative Western blot for carcinoembryonic antigen-related cell adhesion molecule (CEACAM) 1, epidermal growth factor receptor (EGFR), and insulin receptor (IR)-β expression in early endosomal fractions of renal proximal tubule LLC-PK1 cells treated with ouabain (100 nmol/L, 30 minutes). B through D, Corresponding quantitative data as the mean±SEM of 5 experiments. Early Endosomal Antigen-1 (EEA1), an early endosomal marker, was used as loading control. *p<0.05 vs Control. C, control; O, ouabain.

**Figure 4-3.** Representative Western blots and quantitative protein expression shown as the mean±SEM of 5 samples for carcinoembryonic antigen-related cell adhesion molecule (CEACAM) 1 (A and B), epidermal growth factor receptor (EGFR; C and D), and insulin receptor (IR)-β (E and F) expression in early endosomal fractions of renal proximal tubule P11, PY-17, and C2-7 cells treated with exogenous ouabain (100 nmol/L) and/or Insulin (100 nmol/L) for 30 minutes. Early Endosomal Antigen 1 (EEA1)
was used as loading control. *p<0.05 vs Control. C indicates control; I, insulin; O, ouabain; O+I, ouabain+insulin. CEACAM1; IR-β; EGFR.

**Figure 4-4.** Representative images showing the cellular distribution of insulin receptor-β subunit (green fluorescence) and Na/K–ATPase-α1 subunit (red fluorescence) after ouabain (100 nmol/L, 1 hour) and/or insulin (100 nmol/L, 1 hour) treatment in renal proximal tubule LLC-PK1 cells. Merged image shows colocalization. N=5 samples studied from each group.

**Figure 4-5.** Representative Western blots (A) and quantitative protein expression shown as the mean SEM (B) of 8 samples of Na/K–ATPase-α1 subunit expression in early endosomal fraction of renal proximal tubule LLC-PK1 cells treated with ouabain (100 nmol/L) and/or Insulin (100 nmol/L) for 1 hour. Early endosomal antigen 1 (EEA1) was used as loading control. *p<0.05 vs control. C indicates control; I, insulin; O, ouabain; O+I, ouabain+insulin.

**Figure 4-6.** A and C, Representative Western blots for phosphorylated (p-Src) and total Src (c-Src) in LLC-PK1 whole cell lysates and P11 cell early endosomal fractions, respectively. B and D, Corresponding quantitative relative optical densities of p-Src (relative to c-Src) in LLC-PK1 whole cell lysates and early endosomal fractions, respectively, with exogenous ouabain (100 nmol/L) and/or insulin (100 nmol/L) for 30
minutes. Quantitative data shown as the mean±SEM of 5 experiments. *p<0.05 vs control. C indicates control; I, insulin; O, ouabain; O+I, ouabain+insulin.

**Figure 4-7.** A, Representative Western blots for carcinoembryonic antigen-related cell adhesion molecule (CEACAM) 1 and caveolin 1 in renal proximal tubule LLC-PK1 cells treated with exogenous ouabain (100 nmol/L, 1 hour) and/or insulin (100 nmol/L, 1 hour) after immunoprecipitation with anti-Na/K–ATPase-α1 subunit antibody. B and C, Corresponding quantitative data for CEACAM1 and caveolin 1, respectively, as the mean±SEM of 4 experiments. *p<0.001 vs control. C indicates control; I, insulin; O, ouabain; O+I, ouabain+insulin.

**Figure 4-8.** Effect of high-salt diet (2%, 4% or 8%) vs control (0.4%) on systolic blood pressure (mmHg) in mice lacking carcinoembryonic antigen-related cell adhesion molecule 2 (Cc2+/−; □) vs wild-type (WT; □). Data shown as the mean±SEM of measurements performed on 5 animals in each group. *p<0.05 vs WT.

**Figure 4-9.** Effect of high salt diet (8%) on body weight (grams) and urinary sodium excretion (U_Na; microequivalents per day) in mice lacking carcinoembryonic antigen-related cell adhesion molecule 2 (Cc2+/−; ●) vs wild-type (WT; ○). Data shown as the mean±SEM of measurements performed on 5 animals in each group.
Figure 4-1.
Figure 4-2.
Figure 4-3.
Figure 4-4.
Figure 4-5.
Figure 4-6.
Figure 4-7.
Figure 4-8.
Figure 4-9.
Chapter 5

Discussion

There exists a strong correlation between cardiovascular and metabolic complications in patients with chronic renal failure (Tonelli et al., 2006; Lastra et al., 2014). It has also been well established that cardiotonic steroids, including marinobufagenin and ouabain, and insulin are elevated during chronic renal failure, and contribute to hypertension and insulin resistance, respectively, in these patients. An extensive body of work has catalogued three features for the sodium potassium pump: as a pump, as an enzyme, and as a receptor to cardiotonic steroids. In particular, focused research over the past two decades has disclosed a paradigm shift in sodium pump function, highlighting its role as a “signalosome” to facilitate intracellular signaling. For instance, CTS-induced signaling through the Na\(^+\)/K\(^+\)–ATPase includes Src activation, and Fli-1 regulation to increase collagen synthesis contributing to cardiac and renal fibrosis (Elkareh et al., 2007; Kennedy et al., 2008; Fedorova et al., 2009; Haller et al., 2012) and accelerated wound healing (El-Okdi et al., 2008). CTS-induced synthesis of collagen may be one of the factors contributing to vascular dysfunction in preeclampsia. Bagrov has demonstrated that development of preeclampsia is associated with increased placental
MBG levels, and depressed Fli-1 expression that results in markedly elevated collagen-1 expression in umbilical arteries (Nikitina et al., 2009).

There now exists ample evidence to support both of the classic and alternative pathways working in parallel to effect physiological consequences of cardiotonic steroid binding to the sodium pump. Among the well-studied signaling effects of sodium pump is CTS-mediated development of salt-sensitive hypertension and natriuresis. Results of the studies discussed above provide mechanistic details toward the associated developments of insulin resistance and chronic renal failure.

Global null deletion of Ceacam1 in mice caused an increase in blood pressure with increased activation of renal renin-angiotensin systems (Huang et al., 2013). Additionally, there was upregulation of renal (pro-)renin receptor expression via PI3K-Akt activation of cAMP response element-binding protein 1 in the kidney of Cc1-/− mice. Inhibition of PI3K and phospho-Akt attenuated (pro-)renin receptor expression. Mice lacking Cc2-/− also developed increased systolic blood pressure correlating with higher dietary salt consumption. Thus, in the mice, CEACAM1 and CEACAM2 may exhibit functional redundancy in regulating blood pressure. We propose that CEACAM1 could functionally replace CEACAM2, present in mice but not human or rats, in regulating blood pressure with elevated cardiotonic steroids, such as chronic renal failure. Additional studies characterizing the Cc2-/− mouse model would be helpful. Some suggested studies include measurement of endogenous MBG levels, which would be expected to be higher in CEACAM2-deficient mice as compared to WT mice, and contribute to our observed salt-sensitive hypertension.
The salt-inducible kinase (SIK1) is an intracellular protein activated in response to a rise in sodium level, and interacts with the Na\(^+\)/K\(^+\)–ATPase in regulating blood pressure (Popov et al., 2011). Alterations in SIK1 have been reported in animal models of high blood pressure. Some cellular processes related to insulin effects on the kidney and other tissues also appear to involve SIK1. Thus, SIK1 network could represent another “crossroad” in modulating the sodium pump toward the development of impaired blood pressure and insulin regulation (Jaitovich and Bertorello 2010b).

If, as predicted, the incidence of diabetes does indeed increase in the future, associated diseases may also have a greater impact. While a clear clinical correlation between chronic renal failure and associated cardiovascular and metabolic complication has been noted in the literature, our understanding of the mechanistic basis of this association remains deficient. The work detailed in this thesis strongly support an association between CTS-mediated sodium pump and IR signaling via CEACAM protein, and provides valuable information to enhance our understanding of the development of insulin resistance in clinical renal failure patients. If these data are confirmed as proposed above and clinically, they will likely contribute significantly to improved therapeutic outcomes for these patients in the future.
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Appendix A

CEACAM1 Protein Expression in LLC-PK1 Cells

A.1 Loading Curve of CEACAM1 Protein Expression in Early Endosomes of LLC-PK1 Cells.

To determine optimal loading detection of CEACAM1 protein, increasing amounts (7.5, 15, or 30 µg) of early endosomal fraction of LLC-PK1 cells were assayed. Briefly, varying amounts of protein in equal volume were loaded on 4-12% SDS-PAGE gel, and transferred onto nitrocellulose membrane for Western Blot analysis. Blots were probed with polyclonal anti-rabbit CEACAM1 antibody (ab. No 513; 1:1000; secondary anti-rabbit antibody; 1:10,000). Band of 120 kDa corresponding to CEACAM1 was detected in all three lanes, with signal intensity increasing in parallel with protein amount loaded. Figure A-1 shows that CEACAM1 protein was detectable if as little as 15 µg of early endosomal extract was assayed. To ensure any changes in protein expression levels, 50 µg of early endosomal extracts were loaded for subsequent experiments.
A-2 CEACAM1 Protein Expression in Early and Late Endosomes of LLC-PK1 Cells.

We determined if CEACAM1 protein was expressed in early or late endosomal fractions of porcine renal proximal tubule LLC-PK1 cells. Briefly, 50 µg of early or late endosomal fractions in equal volume were loaded, and probed for CEACAM, or EGFR. Both CEACAM1 and EGFR were detectable in early, but not late, endosomal fraction of renal LLC-PK1 cells (Figure A-2). Early Endosomal Antigen 1 (EEA1) and Rab7 are routinely used as markers for early and late endosomes, respectively. Their restricted expressions within the corresponding extractions confirm minimal contamination between early and late endosomal fractions using our isolation protocol.
**Figure A-2.** Representative Western Blots of CEACAM1 and EGFR protein expressions in early or late endosomal fractions of LLC-PK1 cells. Early Endosomal Antigen 1 (EEA1) and Rab7, early or late endosomal markers, respectively, were used as loading control.
Appendix B

Caveolin-1 Protein Expression and Phosphorylation in Early Endosomes of C2-7 Cells

B.1 Relative Expression of Caveolin-1 Protein in Early Endosomes of C2-7 Cells.

We confirmed attenuated caveolin-1 (cav-1) protein expression in early endosomal fractions of porcine renal proximal tubule C2-7 cells with knock-down expression of caveolin-1 (Figure B-1). In comparison, caveolin-1 protein expression was maintained in corresponding control P11 cells, and warrants continued implementation of the endosomal fractionation protocol for subsequent studies.

![Figure B-1](image)

**Figure B-1.** Representative Western Blots of Caveolin-1 protein expressions in early endosomal fraction of renal proximal tubule P11 and C2-7 cells. Cav1–Caveolin-1.
B.2 Relative Src Phosphorylation in Early Endosomes of C2-7 versus P11 Cells.

We determined if phosphorylated Src was detectable in caveolin-1 knock-down C2-7 cells in comparison to control P11 renal epithelial cells. Figure B-2 shows detectable pSrc in P11 cells, but not in C2-7 cells lacking caveolin-1, thereby confirming previous reports of an essential role of caveolin-1 in Src phosphorylation to propagate sodium pump signaling.

![Figure B-2](image)

**Figure B-2.** Representative Western Blots of pSrc and cSrc protein expression in early endosomes of renal C2-7 and P11 cells. cSrc–total Src; pSrc–phosphorylated Src.